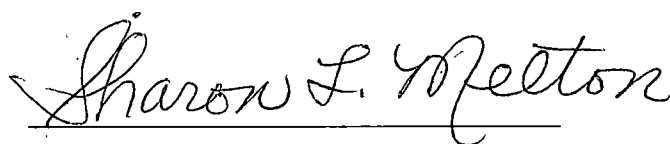


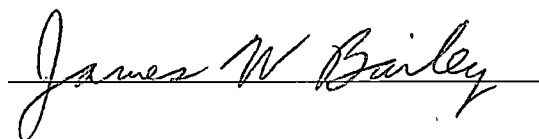
To the Graduate Council:

I am submitting herewith a thesis written by Maïke Johnsen entitled "The effect of dietary fats and supplements on markers of oxidative stress in rats." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with a major in Nutrition.


Dr. D. S. Sachan, Major Professor

We have read this thesis
and recommend its acceptance:


Sharon L. Mecton


James W. Bailey

Accepted for the Council:


Interim Vice Provost and
Dean of the Graduate School

**The effect of dietary fats and supplements on markers
of oxidative stress in rats**

A Thesis

Presented for the Master of Science Degree

The University of Tennessee

Maike Johnsen

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Abstract

Oxidative stress assessed by TBARS, lipid hydroperoxides and protein carbonyls were investigated in male Sprague-Dawley rats. The present study reports the results of three experiments. In the first experiment, rats (n=20) were divided into four groups, a control, exercised, supplemented, and exercised plus supplemented group. The supplement given was a combination of choline, carnitine and caffeine. The supplement significantly increased TBARS and protein carbonyl concentrations in serum. Exercise significantly increased TBARS concentrations in serum but reduced them in testes. Protein carbonyl concentrations were decreased in skeletal muscle and brain due to exercise. In the second experiment, rats (n=24) were fed a diet containing fish oil, corn oil, safflower oil or palm oil as the dietary source of fat. In the animals fed unsaturated fatty acids, the concentration of lipid peroxidation was higher than for the animals fed a saturated diet. Protein carbonyl concentrations were increased with the saturated diet. In the third experiment, rats (n=24) were fed two different diets with predominantly trans or cis fatty acids. Half the animals in each group were killed 2 hours after an acute dose of ethanol. Trans fatty acids reduced oxidative stress measured by the three markers; the acute dose of ethanol had no significant effect. Lipid hydroperoxide concentrations were the least affected by any of the treatments. In conclusion, the effects of different treatments on oxidative stress depend not only on the marker but are also tissue specific.

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Abbreviations

ADH.....	alcohol dehydrogenase
ATP.....	adenosine triphosphate
BHT.....	butylated hydroxytoluene
CCC	choline, carnitine and caffeine supplement
cAMP	cyclic 3',5'-adenosine monophosphate
CoA.....	coenzyme A
CPT-I.....	carnitine-palmitoyl-transferase I
CFA.....	cis fatty acids
DMBA.....	7,12-dimethylbenz(a)anthracene
DMSO.....	dimethylsulphoxide
DNA.....	deoxyribonucleic acid
DNPH.....	2,4-dinitrophenylhydrazine
EDTA.....	ethylene-di-nitolo-tetra-acetic acid
ELISA	enzyme linked immuno assay
FOX.....	ferrous ion oxidation with xylenol orange
HNE	4-hydroxynon-2-enal
HO [•]	hydroxyl radical
H ₂ O ₂	hydrogen peroxide
HPLC	high performance liquid chromatography
GC-MS.....	gas chromatography – mass spectrometry
GPx	glutathione peroxidase

LDL.....	low density lipoprotein
MDA.....	malondialdehyde
MEOS.....	microsomal ethanol oxidizing system
NAD ⁺	nicotinamide adenosine dinucleotide
NADH.....	reduced nicotinamide adenosine dinucleotide
NADP.....	nicotinamide adenosine dinucleotide phosphate
NADPH.....	reduced nicotinamide adenosine dinucleotide phosphate
NS/NE.....	non-supplemented, non-exercised group
NS/E.....	non-supplemented, exercised group
O ₂ ^{•-}	superoxide anion radical
RNA.....	ribonucleic acid
ROS.....	reactive oxygen species
R-PIA.....	R-phenyl isopropyl adenosine
RQ.....	respiratory quotient
S/NE.....	supplemented, non-exercised group
S/E.....	supplemented and exercised group
SDS.....	dodecyl sodium sulfate
SOD.....	superoxide dismutase
TBA.....	thiobarbituric acid
TBARS.....	thiobarbituric acid reactive substances
TFA.....	trans fatty acids
UV.....	ultraviolet

Chapter I

Introduction

At any given time, biological systems are subjected to oxidative stress. Oxygen derived radicals and already damaged molecules are mediators of oxidative damage. Atherosclerosis, cancer and aging are some of the well known examples for a variety of diseases and health situations in which oxidative stress is involved. Often it is difficult to determine if oxidative stress is a cause or a result.

Nutritional factors such as dietary fats have the ability to modulate oxidative stress by enhancing protective mechanism or promoting the production of radicals. Oils high in unsaturated fatty acids are known to increase lipid peroxidation, however, there are other compounds in the oil that are known to have protective action. The reported effects of trans isomers on diseases are inconsistent; results of some studies indicate that trans isomers react similar to saturated fats, but other studies report their behavior similar to their cis isomers.

Endogenous lipids are a second source of fats. The supplement combination of carnitine, choline and caffeine has been shown to reduce body fat and increase β -oxidation, which implies the mobilization of endogenous lipids (Hongu and Sachan 2000). Interestingly, the respiratory quotient did not change, suggesting the transfer of acyl chains onto carnitine and disposal in urine (Sachan and Hongu 2000).

Oxidative stress has been investigated for almost a century and a problem arises in comparing studies due to the wide variety of indices available to study oxidative stress. Various methods are employed, so once a marker is accepted, difficulties in interpreting the results, such as the use of the right pH are often ignored (Ohkawa et al. 1978). Additionally, polyunsaturated fatty acids are easily attacked by free radicals producing lipid peroxidation products. However, the chain length and degree of unsaturation determines the yield of different oxidation products (Visioli et al. 1998). Overall, it is better to measure two or more indicators to take these factors in account. Therefore, in the present study thiobarbituric acid reactive substances (TBARS) as a general indicator for oxidative damage, lipid hydroperoxides as specific biomarkers for lipid peroxidation and protein carbonyls for the degree of protein oxidation were employed.

The objective was to assess oxidative stress in rats fed a variety of diets in our laboratory by other investigators. The questions were: 1) Does fat mobilization caused by choline, carnitine and caffeine supplementation increase oxidative stress? 2) Does the exercise training with or without the supplement affect oxidative stress? 3) Do the types of fat used in a diet modulate markers of oxidative stress differently? 4) Do the cis and trans fatty acids in a diet affect oxidative stress differently?

Chapter II

Literature Review

II.1 Oxygen, Reactive Oxygen Species and Life

Oxidative stress is described as the phenomenon of the imbalance of prooxidants and antioxidants. An increase in prooxidants or a reduction in antioxidants can shift the equilibrium towards prooxidants. This can lead to oxidation of lipids, carbohydrates, DNA, and proteins, and furthermore can damage cells to the degree that cell death occurs. This review is an attempt to discuss factors involved in the oxidative stress mechanisms and some of the metabolic consequences.

It is the paradox of life that oxygen is essential for aerobic life and inherently harmful at the same time. Oxygen (O) is a chemical element that is essential for aerobic metabolism, but it is also a radical. A radical is defined as a molecule that has one or more unpaired electrons on its outer valence shell. Thus, molecular oxygen (O₂), also called triplet oxygen, is a bi-radical because it has two unpaired electrons. However, it is not very reactive but can easily undergo oxidation-reduction reactions.

Most of the oxygen consumed during respiration is metabolized to water as shown in **Figure 2-1**. An enzyme called cytochrome oxidase, the terminal electron acceptor of the electron transport chain, catalyzes the reaction. To allow continuing electron transport it loads four electrons and three hydrogen protons during a tetravalent reduction onto oxygen (Davies 1994). The addition of these four electrons happens in four separate

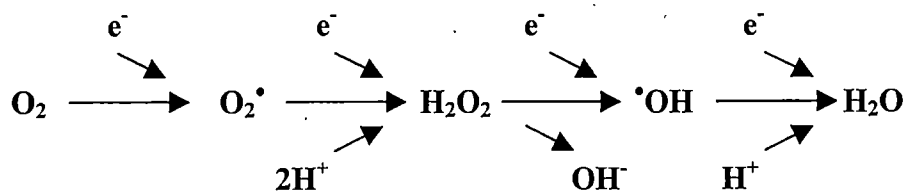


Figure 2-1 At the end of the mitochondrial electron transport chain oxygen functions as an electron dumpster to enable the ongoing ATP production of the electron transport chain. The addition of four electrons and three protons to an oxygen molecule is facilitated by cytochrome oxidase.

steps. However, approximately 5% of oxygen is not completely metabolized to water.

The intermediates, superoxide anion radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$), produced during this chain of reductions can escape and leak out of the electron transport chain (Clarkson and Thompson 2000).

All these intermediates and other oxygen rich molecules are very reactive with other molecules or form upon electron transfer molecules that are more reactive; they are summarized as reactive oxygen species (ROS). However, it has to be understood that not all ROS are radicals; H_2O_2 is not a radical by definition, but because it is able to easily abstract an electron from transition metals to form the highly reactive hydroxyl radical, it is a ROS. In addition, H_2O_2 is able to diffuse into membrane bilayers and bulk lipid depots and therefore can be quite hazardous if not controlled (Ternay and Sorokin 1997).

Besides ROS, other radicals such as reactive nitrogen species (RNS), products of lipid peroxidation or protein oxidation exist that are able to damage other molecules and inherently endanger cells.

Besides the mitochondrial electron transport chain, other controlled and uncontrolled reactions generate H_2O_2 , $\text{O}_2^{\bullet-}$ and other ROS. Approximately 10-15% of oxygen we breathe is not used by the mitochondria. Some oxygen is used to detoxify foreign compounds into less toxic ones and again some is needed for synthesis of several hormones (Gutteridge and Halliwell 1994). Two percent of the total oxygen consumption in the resting state is used to produce H_2O_2 by the mitochondria (Clarkson and Thompson 2000). Peroxisomes generate H_2O_2 under physiologic conditions and so do several cytosolic enzymes such as xanthine oxidase (Ji 1996). Ubiquinone, phagocytic cells, ROS reacting with each other and autooxidation are responsible for a large amount of ROS production (Davies 1994). Thus, the generation of ROS is constantly happening and small amounts of ROS seem to exist at any time.

As ROS are present at all times it is important to consider their metabolic fate. One possibility is the scavenging by antioxidants and concurrent metabolism or removal, which is discussed in the next section. Another is that two radicals bind together and form a non-radical or in the case of two $\text{O}_2^{\bullet-}$ form H_2O_2 , a less reactive molecule. Nevertheless, it is more likely that ROS attack molecules like lipids, proteins or DNA to abstract the needed electron leaving the attacked molecule as the new radical. The most well described form of radical attack is lipid peroxidation and its subsequent chain propagation producing a variety of reactive molecules that can destroy a cell. The propagation of the lipid peroxidation chain is affected by several factors. First, lipids

have to be close enough to be attacked, which is often the case in lipoproteins or membranes in which lipids present a major component. Second, antioxidants present which are able to interrupt the chain reaction. Third, other molecules such as proteins may be attacked instead of a lipid molecule and thus interrupt the chain. Hence, not only ROS but also radicals produced by lipid peroxidation can oxidize proteins. The possible effects of oxidizing proteins include inactivating enzymes, modifying proteolytic activity or cross-links with DNA or other proteins. Probably the least affected, but with the most detrimental outcome, involves DNA. ROS, peroxidized lipids and oxidized proteins are all capable of damaging DNA. DNA strand breaks, protein-DNA cross-links and disruptions of DNA replication are results of oxidative stress.

At any given time, the intact cell is subjected to some oxidative stress, which can be controlled by activating certain protective mechanism of the cell, called antioxidant systems. Thus, intact cells have both antioxidants and prooxidants that are kept at equilibrium, which when disturbed in the favor of prooxidants is defined as oxidative stress (Sies 1991). An introduction to antioxidant mechanisms is presented in the next section.

II.2 Antioxidants

Various other antioxidants are available to protect us from oxidative stress; some of these are summarized in **Table 2.1**, grouped by their physiological function. Antioxidants are defined as substances, which delay or inhibit free radical attacks thereby decreasing oxidative damage to cells. These compounds include enzymes, nutrients and drugs. Not all antioxidants use the same principle for protecting cells from oxidative

Table 2.1

Antioxidants and oxidative repair systems of physiological importance

Enzymes

Glutathione peroxidase
Glutathione reductase
Glutathione-S-transferase
Catalase
Superoxide dismutase
Phospholipase A₂

Nutrients

Tocopherols and tocotrienols
Ascorbic acid
 β -Carotene and carotenoids
Ubiquinone
Manganese

Transition metals as cofactor in proteins

Copper
Zinc
Iron
Selenium
Flavonoids

Drugs

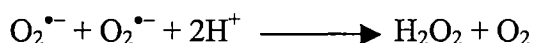
Salicylate
Xanthine oxidase inhibitors
DMSO

Others

Thiol-groups in amino acids
Glutathione
Ceruleplasmin
Bilirubin
Ferritin
Transferrin
Uric acid

damage: Some antioxidants are scavenger. They pick up reactive oxygen species or they regenerate other antioxidant species by losing their own activity. Examples are vitamin E, vitamin C, ubiquinol and uric acid. Another protective mechanism is the metabolism to less reactive forms by superoxide dismutases (SOD), oxidases, catalase, and glutathione peroxidase (GPx). Moreover, once damaged molecules are present, they need to be repaired or removed and replaced to prevent further damage.

The most important and first lines of defense against reactive oxygen species are antioxidant enzymes such as SOD, catalase and GPx. They can be activated or up regulated individually depending on the basal activity and substrate concentrations, thereby can increase with increasing levels of reactive oxygen species. Perhaps the most investigated enzymes in regard to oxidative stress are SOD's, their function is to catalyze the reaction of two superoxides to form H_2O_2 .



Although H_2O_2 is not a radical, it is still toxic for the cell and is readily detoxified by GPx or catalase to water. However, their affinity to H_2O_2 is different, GPx acts at low concentrations and catalase acts at high concentrations. Indeed, this explains why the latter seems to be mostly active in peroxisomes and GPx is responsible for removing H_2O_2 in the cytosolic fraction where generally less H_2O_2 is produced (Davies 1994).

Antioxidant nutrients are naturally occurring in foods of plant and animal origin but cannot be synthesized by the human body. Consequently, they have to be taken in through the diet; vitamin E (tocopherols and tocotrienols), vitamin C (ascorbic acid) and the provitamin β -carotene are the major antioxidants. Several authors propose to count glutathione and some transition metals listed in **Table 2.1** as antioxidant nutrients

because they are essential for several enzymes and proteins involved in the regulation of oxidative stress (Davies 1994, Ternay and Sorokin 1997). Transition metals like copper, zinc and manganese are essential for SOD. Iron is a cofactor in catalase and selenium in GPx. Ferritin and caeruloplasmin are proteins responsible for storing and transporting transition metals, iron and respectively copper. They bind the transition metals tightly to avoid reduction/oxidation reactions, which would produce highly reactive radicals. Thus, some literature refers to them as antioxidants proteins

Vitamin E is the major, lipid-soluble antioxidant found in the cell membranes protecting not only fatty acids in the membranes but also free fatty acids. Vitamin E is able to give away its proton to interrupt the chain reaction of lipid peroxidation. Several study results indicated that vitamin E deficiency is correlated with higher levels of lipid peroxidation, and that vitamin E supplementation reduced lipid peroxidation (Davies et al. 1982, Harats et al. 1990, Hu et al. 1989). For example, when a diet was consumed, which contained 15% polyunsaturated fatty acids, DNA damage was prevented with an additional vitamin E supplement of approximately 70 mg (Jenkinson et al. 1999). In recent years, focus on vitamin E studies has been on many of its derivatives, especially tocotrienols, which have been shown to be more effective antioxidants than tocopherols (Kamat and Devasagayam 1995, Serbinova et al. 1991).

Ubiquinone, sometimes called Coenzyme Q, is another lipid soluble antioxidant. Although the exact mechanism is still unclear, it is speculated that ubiquinone acts by inhibiting and interrupting lipid peroxidation as radical scavenger or by regenerating reduced vitamin E (Ternay and Sorokin 1997). Physical activity known to increase lipid peroxidation also increased ubiquinone concentrations in rat liver. Similarly, more

unsaturated fatty acids in the diet increased liver lipid peroxidation and ubiquinone levels in rats. The investigators suggested that this could be due to a transfer of ubiquinone from blood to tissue as it has been shown in earlier studies that ubiquinone increased in tissue in parallel to a decrease in blood (Quiles et al. 1994).

Often β -carotene is considered a lipid soluble antioxidants. It is well known as an effective quencher of singlet oxygen, a ROS, which is easily converted to $\text{OH}^\bullet$. It is suggested that other carotenoids may have some antioxidant capacities, but evidence has yet to be found.

Vitamin C has several metabolic roles and even as an antioxidant, it has different approaches to prevent oxidative damage. In the aqueous phase of extracellular fluids and within cells vitamin C is able to directly scavenge radicals and is able to interact with vitamin E radicals in membranes to reduce these. Pre-eclampsia is associated with an increase in plasma lipid peroxidation and reduced vitamin C levels. The low vitamin C levels are suggested to result from its action as a radical scavenger and vitamin E regenerator (Bowen et al. 1998). Several studies suggest that vitamin C helps reduce the risk of cancers, coronary heart disease and other age related diseases. Supplementary vitamin C acts as antioxidant against lipid peroxidation and protein oxidation (Barja et al. 1994). Vitamin C supplementation successfully prevented increase of lipid peroxidation products in plasma of smokers (Harats et al. 1990). Recent reviews focused on the importance of vitamin C as an antioxidant (Carr and Frei 1999, Jacob and Burri 1996).

Glutathione is classified as an antioxidant because it exhibits some antioxidant potential by regenerating oxidized vitamin C. In giving away its hydrogen, it can also prevent further radical production by reducing H_2O_2 to water. Additionally, it is

suggested that glutathione is able to repair DNA radicals by giving away its hydrogen from the thiol-group (Gutteridge and Halliwell 1994).

Most of the previously mentioned antioxidants have their role in interrupting oxidative processes or preventing these by scavenging reactive oxygen species that will otherwise damage lipids, proteins or DNA. Additionally, there are compounds that reverse oxidative damage, directly or indirectly. Disulfide reductases are an example for direct repair systems. They are able to break disulfide bonds within or between proteins. Another example is methionine sulfoxide reductase, which can reactivate oxidized methionine residues of proteins. Direct repair systems have yet to be fully understood, whereas indirect repair systems are far better understood. Indirect repair systems are responsible for recognizing, removing, and replacing damaged molecules. An example is the lipid peroxidation chain reaction where an intact lipid is attacked by a damaged lipid to form a lipid hydroperoxide for faster decomposition. Phospholipase A₂ is an enzyme activated by lipid hydroperoxides to remove them from the membrane keeping the membrane intact and stopping the propagation of lipid peroxidation. Without this repair system, the membrane would lose its integrity and it could lead to increased oxidation of proteins and DNA. Proteases are responsible for recognizing oxidized proteins and degrading them to amino acids. The undamaged amino acids are recycled for synthesis of new proteins whereas the oxidized amino acids are eliminated. There is evidence, summarized by Davies (1994), that several enzymes are available for DNA repair such as endonucleases and that an intracellular system is responsible for excision and repair.

II.3 Factors Influencing the Status of Oxidative Stress

As oxidative stress is a constant byproduct of life, the balance between antioxidants and oxidants is the best possible protection. Anything that will affect either one will affect health, well-being or therapeutic outcome. In **Table 2.2**, some of these factors are listed to illustrate the broad range of possible reasons for an increase in oxidative damage. A few of these are closely examined here.

Naturally, anything that enters the body, whether it is through breathing, eating or drinking, has an impact on our system. Eating a diet with lots of fruits and vegetables, which are rich in antioxidant nutrients, may help improve our plasma antioxidant defenses, which may be reflected in cellular antioxidant concentrations. Deficiency of any nutrient involved in the antioxidant defense mechanisms would significantly affect the balance between oxidants and antioxidants. To prevent a deficiency consumers often chose to take so-called complete supplements to add vitamins and minerals, whether they need it or not. The supplements taken may also include transition metals. While these metals are beneficial and necessary, they might pose a risk if they occur beyond adequate levels and begin to be present in unbound form in plasma. Free transition metals readily give away a hydrogen proton and the new reduced form is highly reactive and may produce free radicals. Dietary fats and alcohol can also influence the antioxidant status. High intakes of both, occasionally or on a regular basis are known to cause increased oxidative stress. They are discussed in section II.6.4 and section II.6.6. On the other hand, a high protein diet in adult rats did not affect oxidative stress (Petzke et al. 2000).

Tobacco smoke, soot, and exhaust fumes are known to contain a considerable amount of free radicals. Other environmental pollutants in the air and food are able to

Table 2.2

Possible causes for an increase in oxidative damage in the human pathophysiology

Age
Enzymes
 Xanthine oxidase
 Cytochrome P-450
Mitochondrial electron transport
Glucose autooxidation
Diet
 Polyunsaturated lipids
 Antioxidants
 Transition metal ions
Cigarette smoke
Alcohol
Environmental toxins
Exercise
Radiation
Inflammation
Ischemia/Reperfusion
Prescription drugs
 Acetaminophen (Tylenol®)
 Paracetamol
 Anthracycline antibiotic doxorubicin

produce oxidative stress, e.g. aluminum, ozone, nitrogen dioxide, sulphur dioxide, ultraviolet radiation to name only a few (Halliwell and Gutteridge 1989, Davies 1994). Although prescription drugs are generally taken to cure or improve a disease condition, sometimes a drug itself or its metabolism can produce free radicals leading to oxidative damage.

Any type of tissue injury by heat, infection, trauma, ischemia, or radiation will increase the blood flow and enable neutrophils to leak into the inflamed area to attack the cause of inflammation. When activated these neutrophils and other phagocytes produce free radicals. Chronic inflammation as found in rheumatoid arthritis or inflammatory bowel disease produces severe oxidative damage and may lead to destruction of joints and parts of the gut (Gutteridge and Halliwell 1994). Omar et al. (1991) reviewed the causes and consequences of reperfusion-ischemia injury. Reperfusion following ischemia can lead to significant more free radical formation, not only by neutrophils but also by xanthine oxidase and other enzymes. During oxygen deprivation, xanthine dehydrogenase is in part converted to xanthine oxidase and once oxygen is available, it will produce large amounts of radicals.

The effect of exercise on markers of oxidative stress is different depending on the type of exercise. While regular exercise has been shown to improve antioxidant defenses, sudden exercise was shown to have the opposite effect. This is discussed in depth in section II.6.5.

Aging is a major concern these days and it is accepted that cumulative oxidative stress is a major contributor. How oxidative stress is related is still debated. However, studies found an increased maximum life span, reduced oxidative damage, and a

reduction of the metabolic rate with caloric restriction. It is suggested that due to a lower metabolic rate less prooxidants are produced that could increase the life span (Sohal and Weindruch 1996).

II.4 Diseases and Pathological Changes Linked to Oxidative Stress

As several metabolic pathways constantly produce free radicals, it is not surprising that there are times when antioxidants and prooxidants are out of balance. In the previous section, it was mentioned that some dietary factors might cause an increase in oxidative stress. In addition, aging is thought to be reason for an inability of sufficiently dealing with oxidative stress, hence oxidative damage increases. Several genetic disorders have been shown to have significantly higher levels of oxidative stress

Table 2.3

Disease states linked to oxidative stress

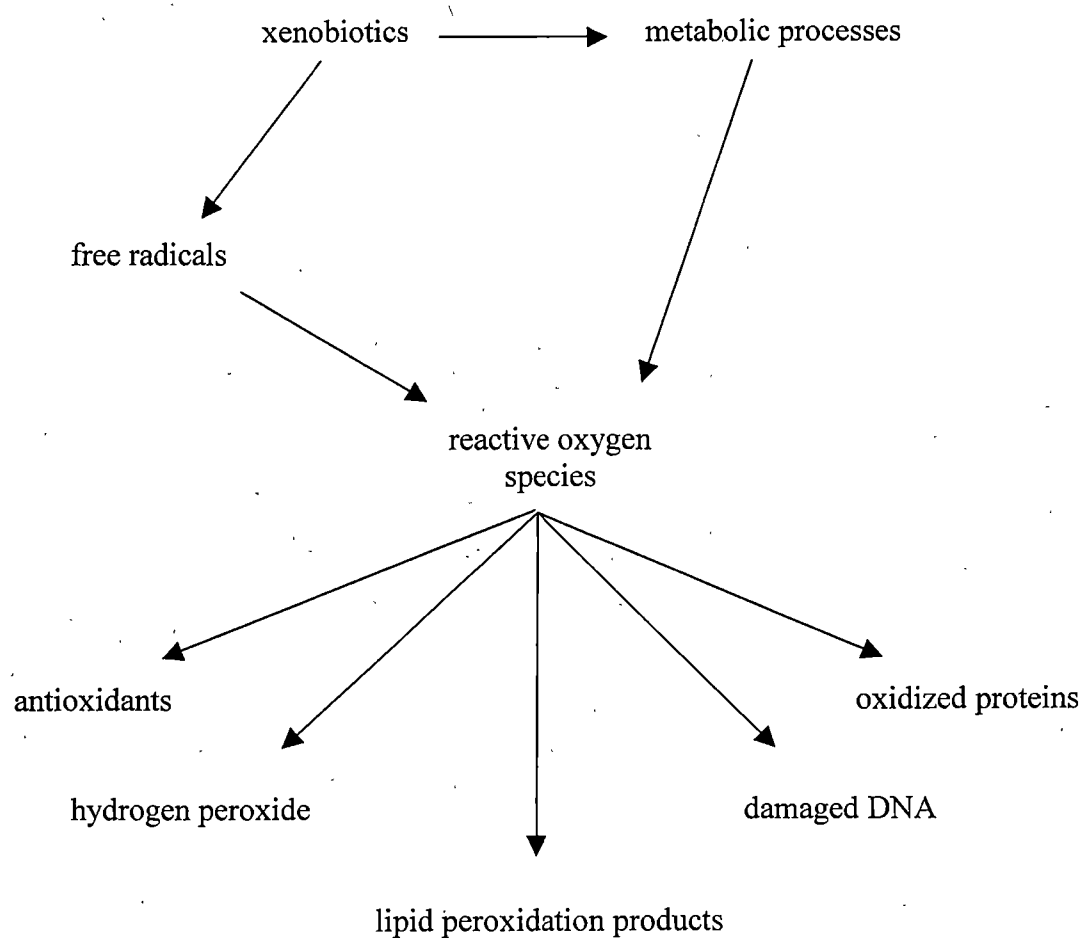
Diabetes mellitus
 Ischemia/ Reperfusion injury
 Myocardial infarction
 Carcinogenesis
 Atherosclerosis
 Alzheimer Disease
 Parkinson Disease
 Down's syndrome
 Rheumatoid arthritis
 Iron overload
 Radiation injury
 Multiple sclerosis

markers. On the other hand, treatments such as radiation therapy will lead to occurrence of more free radicals. In fact, several diseases go hand in hand with increased oxidative damage, but often it is hard to tell what was there first, the disease itself or oxidative stress. For example, in the case of Parkinson's and Alzheimer's disease the exact role of oxidative damage is still under investigation. **Table 2.3** lists some diseases, which are linked one way or the other to oxidative stress; their relationships have been described (Halliwell and Gutteridge 1989, Sies 1991) and are beyond the scope of this review.

II.5 Detection and Measurement of Oxidative Stress in Biological Systems

II.5.1 Overview

Biomarkers of oxidative stress include ROS, antioxidant systems, intermediates and metabolites of reactions with macromolecules; an overview is given in **Figure 2.2**. ROS have short half-lives and are therefore difficult to measure; the antioxidants are categorized in enzymes and nutrients and it depends on several factors if they are good indicators in a specific study. Antioxidant nutrients are often used in studies investigating affects of deficiencies or supplementation on oxidative damage. Antioxidants are measured by their concentration or in the case of antioxidant enzymes by their activity. There are varieties of assays that measure specific non-enzymatic antioxidants or total antioxidant capacity; a comparison can be found in a recently published review (Prior and Cao 1999). Often antioxidant enzymes are measured combined with non-enzymatic antioxidants and other biomarkers to give credit to their interactive behavior as mentioned in section II.2. This is especially important when samples are stored, as it may



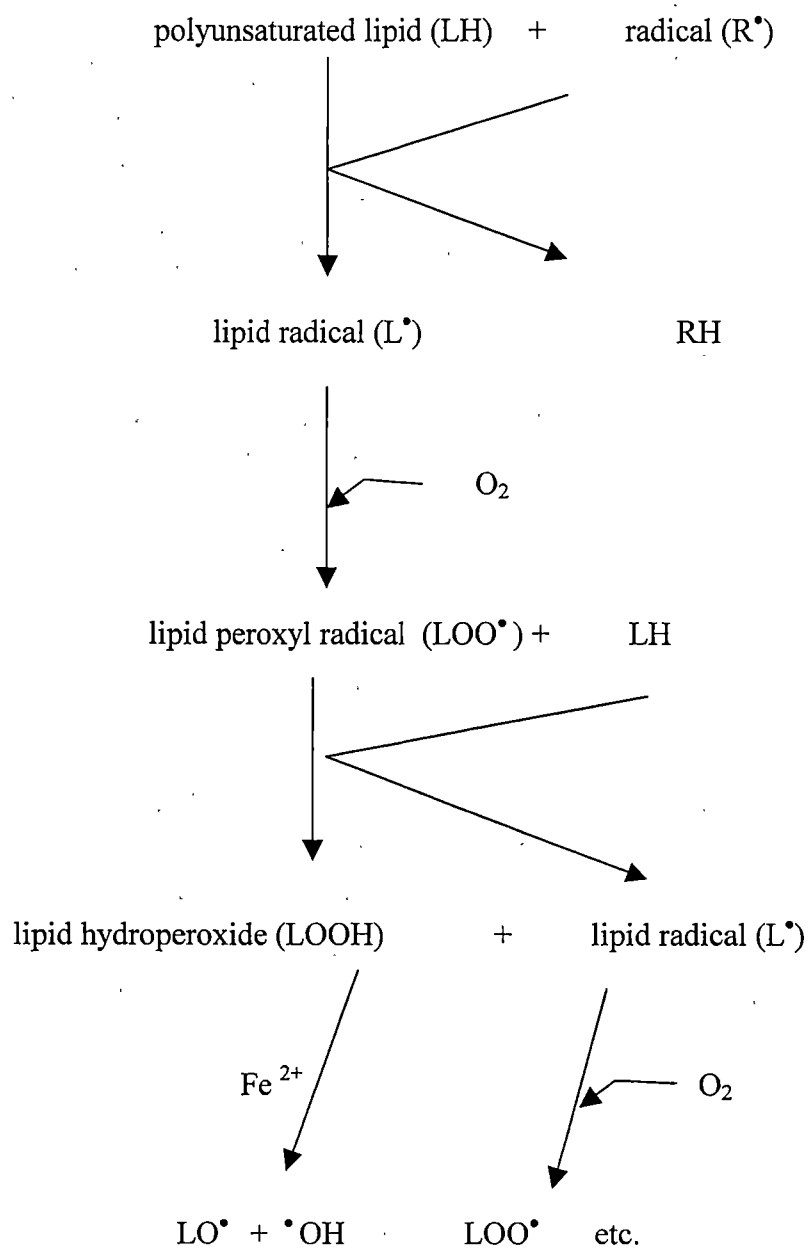
Modified from de Zwart et al. 1999

Figure 2-2 Origin and products of free radical damage

influence some parameters. Free radical damage results in three main groups: peroxidized lipids, oxidized proteins and damaged DNA. Not all, but some of these products are stable metabolites and may be measured in body fluids, breath, urine and/or tissues.

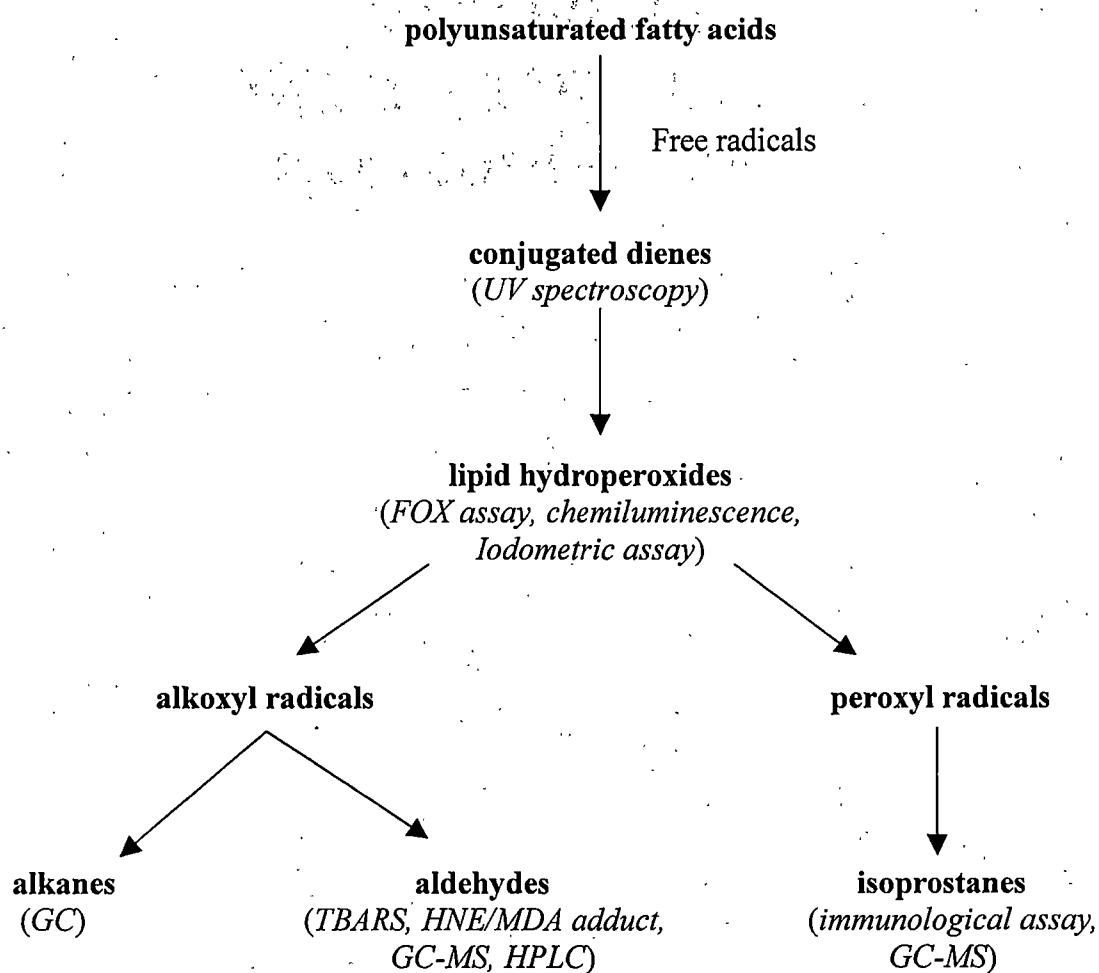
Lipid peroxidation products are studied probably the most. As lipids found in membranes they represent an easy target for radicals and often are the first molecules attacked and damaged. Once oxidized, lipids propagate a chain reaction of lipid peroxidation shown in **Figure 2.3**. Moreover, they enhance oxidation of DNA and proteins. The most common assay for lipid peroxidation is the thiobarbituric acid (TBA) test. Although some researchers warn of interpreting the results of TBA assays, it is used as a kind of gold standard for comparing new assays and estimating the degree of lipid peroxidation. As some investigators doubt the specificity of the test for lipid peroxidation, it is sometimes used to get an overall idea of oxidative stress. Validation of other indices is often done by comparing results with outcomes of the TBA test. However, this practice seems rather questionable, as it is doubtful that all causes of oxidative stress will damage the same molecules to the same degree. Other biomarkers of lipid peroxidation are conjugated dienes, lipid hydroperoxides, aldehydic products, volatile alkanes and isoprostanes; an overview is given in **Figure 2.4**.

Conjugated dienes are defined through the location of their double bonds, which are separated only by a single bond compared to the original double bonds separated by a methyl group. The loss of hydrogen changes a lipid into lipid peroxy radical and the subsequent re-arrangement of the double bonds in one of the fatty acids is the first measurable evidence of lipid peroxidation. Typically, conjugated dienes are measured by UV spectrophotometric techniques at their characteristic absorption maximum of 234 nm.



¹Adapted from de Zwart et al. 1999

Figure 2-3 Schematic proceeding of lipid peroxidation chain reaction¹



¹Modified from Moore and Roberts 1998

Figure 2-4 An outline of lipid peroxidation intermediates and products formed and common assays used to measure lipid peroxidation.¹

A drawback of the techniques is that many solvents produce a background at the same wavelength. Consequently, conjugated dienes are more often measured in experiments documenting absorption changes in a dynamic assay rather than for quantitative determinations (Recknagel and Ghoshal 1966). The peroxy radical attracts a hydrogen proton to form a hydroperoxide, the hydrogen is often abstracted from another lipid molecule, hence enables the propagation of the lipid peroxidation chain reaction. A more in depth introduction of hydroperoxides follows in section II.5.3, as it is a biomarker used in this study.

Hydroperoxides may further be metabolized to aldehydes. The aldehydic products include a wide variety of alkanals, alkenals, alkanes, alkenes and dicarbonyls. Common markers are 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). Compared to free radicals aldehydes are stable, which gives researchers the opportunity to measure them. On the other hand, this stability is a disadvantage as they are able to diffuse through membranes and thus can attack targets distant to their origin. MDA is the most widely used indicator of peroxidation and is probably the most controversial, too. Often MDA is assayed employing TBA, which is further explained in the section II.5.2. Other common assays use HPLC followed by absorbance readings or GC-MS determination, but artifacts always impart a problem when estimating aldehydes.

Measuring volatile alkanes in breath is a non-invasive method, therefore of great interest for human studies. Two alkanes, pentane and ethane, are both derived from unsaturated fatty acid peroxidation and have been found to be correlated with increased TBARS and reduced vitamin E levels. A major problem of measuring alkanes is that they are present in air and may be inhaled. The fraction of inhaled alkanes in exhaled breath is

expected to be 50% or more. In addition, the techniques available are complex, sometimes not very specific, time consuming and may lead to further contamination.

Isoprostanes have been recently discovered and shown to be very promising as markers of oxidative stress. They are peroxy radicals of arachidonic acid esters and subsequently form prostaglandin like compounds. They circulate in plasma and are excreted through urine, therefore represent another non-invasive biomarker. Two techniques, a GC-MS method and an enzyme immunoassay are available, both having the disadvantage of requiring a laborious pretreatment. However, isoprostanes have been successfully measured in biological fluids and tissue of animals and humans. It has been demonstrated that isoprostane F_{2a} is an abundant biomarker in urine that exhibits low variability (Praticò et al. 1998). Morrow and Roberts (1997) suggested that some isoprostanes may also participate in oxidative damage, because earlier studies showed that at least two of the isomers are not only markers of oxidative damage but also exert biological activity and are potent vasoconstrictors.

Proteins may also be attacked by ROS; until now, protein oxidation can only be measured in tissues and plasma. Future investigations have to show whether specific products or metabolites of protein carbonyls are present in urine as well. As protein carbonyls are determined in this study, they are discussed in depth in section II.5.4.

Any type of oxidative damage can have tremendous effects on cell metabolism and cell viability. DNA damage in particular is considered a major factor in the aging process and cancer. Mutations, DNA strand breaks, base release and cross-links with proteins are all results of oxidation processes that occur at all times. Endonucleases and glycosylases repair DNA rapidly by excising the damaged base or deoxynucleotide,

metabolizing and excreting it in urine. As biomarkers of DNA damage uric acid, damaged purine and pyrimidine bases may be measured in urine. They are also found in a variety of tissues or cells. Techniques described include an immuno affinity technique for urine samples (Shigenaga and Ames 1991) and solid phase extraction followed by HPLC with electrochemical detection, the latter was successfully used for urine and mononuclear cells (Dandona et al. 1996, Pilger et al. 1997).

Along with these major biomarkers, several other products, hydrogen peroxide, xenobiotics, and genes are used as indicators of oxidative stress. From an introduction of these is being differed here to reviews about oxidative stress markers (De Zwart et al. 1999, Jenkins 2000, Moore and Roberts 1998).

II.5.2 Thiobarbituric Acid Reactive Substances

After the discovery that tissue suspensions produce a red-pink colored compound with 2-thiobarbituric acid (TBA) under aerobic conditions, further investigations detected an increase of the compound in the presence of ascorbic acid (Bernheim et al. 1948). Subsequently, it was found that autooxidation of certain lipids produce the same substance and ascorbic acid acts as a mere catalyst. Moreover, unsaturated fatty acids were found to be the origin and most likely three-carbon compounds, aldehydes or ketones, formed the still unknown complex with TBA (Wilbur et al. 1949). Follow-up studies focused on identifying the compound and found a few possible candidates, one of them was MDA. Additional experiments with milk fat, MDA, aerobic tissue preparation and oxidized fatty acids revealed an identical color with the same absorption maximum at

532 nm. At this point, it was already obvious that MDA was not solely accountable for the color development (Patton and Kurtz 1951).

Other studies identified the same color complex when rancid fish oil or pyrimidines were used. Although the exact mechanism was still obscure, with 1,1,3,3-tetraethoxypropane (TEP) or 1,1,3,3-tetramethoxypropane (TMP) as MDA standard the red-pink compound was identified as a condensation product of two molecules TBA and one molecule MDA (Sinnhuber et al. 1958). Further studies trying to validate previous results were able to show that TBA products were linear to peroxide values and conjugated dienes, both used in many studies to verify autooxidation. Furthermore, a higher degree of unsaturation, three or more double bonds, increased the yield of the TBA complex expressed in nmol MDA. It was suggested that the unsaturation is an important factor to enable intramolecular rearrangement in the fatty acid followed by subsequent formation of aldehydes and other breakdown products (Dahle et al. 1962). Consequently, this new determination of lipid peroxidation was employed with other substrates.

Because of the experimental procedure, protein present in samples precipitated out and interfered with the readings. The addition of a pyridine-butanol solvent mixture for extraction of the pigment eliminated the problem. After centrifugation, the pigment was found in the lipophilic solvent free of interfering material and could be determined directly via spectrophotometry. This variation allowed the use of the method without interference with up to 20 mg protein and/or 15 mg lipid present in the incubation mixture (Placer et al. 1966).

Nevertheless, other questions were still unanswered. One of them was why some researchers failed to show TBA color production with linoleic acid hydroperoxides. Small changes in pH were found to be responsible. TMP, the control, was not affected by pH changes between 1 and 4.5, but fatty acid hydroperoxides reacted best at a specific pH. Linoleic acid was found to have its maximum absorbance at pH 4 and decreased extremely towards pH 3, the optimum pH to measure linolenic and arachidonic acid hydroperoxides (Ohkawa et al. 1978). This could have significant importance in interpreting results, but seems to be of small concern for most investigators.

In the meantime, sensitivity and specificity of the TBA assay for MDA was questioned and investigated. Using HPLC, which is able to distinguish between different complexes it was found that only about 10% of the pigment is TBA-MDA derived and thiobarbituric acid reactive substances (TBARS) became the more appropriate name (Kosugi et al. 1989). Nonetheless, TBA-MDA has been shown to produce a much higher absorption than other TBA complexes at the same wavelength. It was estimated that over 99% of the absorption was due to MDA representing about 50% of the color produced with the method employed (Esterbauer and Cheeseman 1990). Various other methods are developed to determine TBARS including GC-MS and HPLC techniques (Li and Chow 1994, Luo et al. 1995); some of them are able to detect specific complexes like TBA-MDA. HPLC separation can be introduced before or after adding TBA, depending if free MDA or total TBA-MDA is measured or different TBA reactants were to be classified (Draper and Hadley 1990).

Depending on the method, it has to be considered that lipid peroxidation products are reactive and could act as oxidants during the assay and produce more lipid

peroxidation (Ohkawa et al. 1979, Moore and Roberts 1998). A possible solution is the addition of BHT (butylated hydroxytoluene) to the assay, but studies are not consistent in its use. Jiang et al. (1991) found a significant decrease of TBARS and lipid hydroperoxides when using 80 μM instead of 5 μM BHT. Another group found that a concentration below 3 mM BHT would not be sufficient to prevent further peroxidation during the TBARS assay (Jentzsch et al. 1996). Several studies mentioned the use of BHT but did not give accurate concentrations. Thus, it is difficult to determine how much of the BHT used had a protective effect. However, Jiang et al. (1991) demonstrated that in the TBA assay they employed no lipid hydroperoxide were metabolized to TBARS. Thus, TBARS measured by the TBA assay are present in the tissue and are not produced from lipid hydroperoxides during assay procedure.

II.5.3 Lipid Hydroperoxides

Lipid hydroperoxides are another group of lipid peroxidation products. The method often used to detect lipid hydroperoxides is the iodometric assay, which uses the ability of peroxides to oxidize iodide to iodine, but it lacks sensitivity and specificity. Chemiluminescence based HPLC detection has the advantage of being able to separate different groups of hydroperoxides and is very sensitive, but HPLC or a GC-MS analysis is a time consuming method due to required sample purification steps. Other techniques have taken advantage of enzymes such as glutathione peroxidase and cyclooxygenase. Glutathione peroxidase reduces free hydroperoxides by oxidizing glutathione; glutathione is then reduced again consuming NADPH and the disappearance of NADPH is measured. The activity of the cyclooxygenase may be stimulated with only trace amounts of

peroxides, thus it is a very sensitive method. A disadvantage is that all these methods is that they are either laborious or have low sensitivity (Gutteridge and Halliwell 1990).

A completely new approach was pioneered in the 1980's after Gupta (1973) described a method to detect micromolar concentrations of H_2O_2 using ferrous ions, titanium, and xylenol orange. The reaction is based on the ability of peroxides to oxidize ferrous to ferric ions selectively in dilute acid (Equation 1). The orange sodium salt xylenol orange (XO) in the mixture selectively binds to ferric ions and forms a stable blue-purple complex with an absorbance maximum at 560 nm (Equation 2).



The method was then used to determine how far H_2O_2 production and glucose autoxidation are associated with protein damage. Evidence was found that protein damage is caused by H_2O_2 during glucose autoxidation rather than by the incorporation of glucose into protein (Hunt et al. 1988). Later sorbitol was found to enhance the color signal, which is then stable for a few hours (Jiang et al. 1990). The reason for the color enhancement is that a chemical like sorbitol is able to scavenge hydroxyl radicals to yield hydroperoxyl radicals. As the production of hydroperoxides is preceded by hydroperoxyls and sorbitol regenerates hydroperoxyl radicals, the ferric ion formation is subsequently increased.

After the findings that the assay was sensitive enough to detect very small amounts of hydrogen peroxide, it was compared to the iodometric and the TBA assay to

evaluate its accuracy in determining lipid peroxidation by measuring lipid hydroperoxides. The results were comparable to the iodometric assay and showed that the TBARS underestimated lipid peroxidation (Jiang et al. 1991). Due to sorbitol, a chain reaction of further peroxidation is likely in samples containing a large lipid fraction. This can be avoided by adding BHT, a chain breaking antioxidant to the assay if high levels of lipids are present (Wolff 1994). Although this method is straightforward, simple and sensitive surprisingly few studies have used it.

II.5.4 Protein Carbonyls

Oxidative damage to proteins includes damage to enzymes and other important cell components. Oxidative modification of proteins could be due to glycolysation, formation of Schiff bases, ROS, lipid peroxidation products, or most often metal catalyzed oxidation.

Although metal catalyzed oxidation is not fully understood, a proposed mechanism is that ferrous iron is reduced to ferric iron, often with subsequent formation of H_2O_2 , and then binds to a metal binding site of the protein. The protein-iron complex reacts with H_2O_2 to generate radical species, which generally attack an amino acid side chain close to the metal binding site (Stadtman and Oliver 1991). The modifications include cross-links, aggregation, fragmentation, and molecular changes such as formation of ketones or aldehydes often producing protein carbonyls. Some indicators of these changes are a decreased isoelectric point and changes in molecular weight in damaged proteins compared to undamaged (Davies 1987).

Additionally, enzymes are inactivated and protein degradation is increased after proteins are exposed to free radicals (Davies and Goldberg 1987). Investigations with oxidized glutamine synthetase show that only one histidine residue in the subunit of the enzyme had to be altered to inhibit catalytic activity (Rivett and Levine 1990). A reason could be that enzymes have to bind divalent cations at specific binding sites to activate the enzyme. One altered histidine residue would be able to inhibit the binding as it presents a missing binding site.

In vitro studies indicated that specific proteases degrade oxidized proteins more rapidly and some proteases seem to specifically degrade the oxidized forms of proteins (Lee et al. 1988, Rivett 1985). Stadtman (1990) proposed that a 'marking' of the oxidized protein causes the increased susceptibility to degradation by proteasomes. It is suggested that site-specific alteration introduces a carbonyl group into the protein. Interestingly the studies with glutamine synthetase showed that the susceptibility to degradation increased only when a second histidine residue was oxidized (Rivett and Levine 1990). A few oxidized proteins seem to function differently. One example is two glucose-6-phosphate dehydrogenase form cross-links caused by 4- hydroxy-2-nonenal and are then less susceptible to degradation compared to the undamaged form. The cross-linked form has been also shown to inhibit the multi-catalytic protease responsible for degradation (Friguet et al. 1994). An accumulation of these products could be the reason for the accrual of oxidized proteins in aging and disease.

A carbonyl assay implicates the measurement of oxidized proteins, which are more susceptible to proteases, thus a protease inhibitor cocktail has to be added to the assay. Moreover, nucleic acids have a close absorbance maximum and can interfere with

correct readings. Several assays for protein carbonyl determination are available using borohydride, dinitrophenylhydrazine or gel electrophoresis (Levine et al. 1990). Only recently, methods have been described using ELISA, HPLC or Western blotting (Buss et al. 1997, Levine et al. 1994). In the present study, a spectrophotometric technique using dinitrophenylhydrazine was employed to determine protein carbonyls (Reznick et al. 1994).

II.6 Dietary Factors and Oxidative Stress

There are numerous dietary factors, which have both positive and negative effects on oxidative stress. Some of these are well known antioxidants mentioned earlier in this review. Some other factors are introduced below and are pertinent to the studies reported in this thesis.

II.6.1 Carnitine

L-Carnitine, β -hydroxy- γ -N-trimethylaminobutyrate, is essential for fatty acid metabolism and acyl-coenzyme A (CoA) homeostasis. Mammals are able to endogenously synthesize carnitine from lysine and methionine, and therefore it is not an essential vitamin. Synthesis occurs mostly in liver and kidney, whereas carnitine storage is mostly in skeletal and heart muscle with small amounts found in plasma and other tissues. However, the typical western diet contains carnitine and 54-87 % of the ingested carnitine, the majority from animal foods, is absorbed in the small intestine (Rebouche and Seim 1998).

Carnitine is essential for the function of mitochondrial fatty acid oxidation. Long-chain fatty acids have to cross the mitochondrial membrane to participate in fatty acid oxidation, but can only do so when esterified with carnitine. Within the mitochondria, the fatty acid esters are then transesterified to CoA for β -oxidation. Under normal conditions acyl-CoA esters are further metabolized and regenerate free CoA to participate in several other cellular pathways. It is suggested, that carnitine can act as a buffer freeing CoA by transferring acyl groups onto carnitine if the other pathways are impaired or overloaded (Rebouche and Paulson 1986). Strenuous exercise has been shown to increase acylcarnitine and simultaneously decrease carnitine (Harris et al. 1987). Consequently, both free and esterified carnitines are found in biological systems with acetylcarnitine often being the predominant form.

Acetylcarnitine is able to affect age related changes in the mitochondria in various ways (Shigenaga et al. 1994). One example is age related decline in cardiolipin, an important cofactor of several mitochondrial transport proteins and cytochrome c oxidase. The reduction of cardiolipin may impair the membrane fluidity because of decreased synthesis and increased oxidative stress. Shigenaga et al. (1994) believe that acetyl-L-carnitine is able to improve the metabolic efficiency in mitochondria, therefore decreasing mitochondria derived oxidants. When old rats fed acetyl-L-carnitine, they had some reversal of an age-related decline in the membrane potential and a marked increase in cardiolipin in liver cells (Hagen et al. 1998). However, the rats had a higher rate of oxidant flux and reduced antioxidant levels measured by glutathione and ascorbate, indicating that although the metabolism increased the efficiency of electron transport did not improve. The ability of acetyl-L-carnitine to reverse these age related effects has been

attributed to improved transport of fatty acids into the mitochondria for β -oxidation and subsequent delivery of acetyl-CoA to the tricarboxylic acids cycle (TCA cycle).

However, carnitine failed to change the respiratory quotient (RQ), but increased acetyl carnitine excretion and VO_2max (Daily et al. 1998). It is suggested that acetyl and short chain acyls are being transferred onto carnitine and rapidly leave the skeletal muscle.

Thus, acetyl-CoA accretion in skeletal muscle is minimized and probably adverse effects on cellular metabolism are limited (Brass and Hiatt 1998, Sachan and Hongu 2000).

L-propionyl carnitine has been found to exert a protective effect on heart during reperfusion; it noticeably improved the ratio of glutathione and oxidized glutathione (Ferrari et al. 1989) and a better functional recovery in the myocardium (Leipälä et al. 1991). An indirect antioxidant effect is postulated, possibly by a radical scavenging activity (Packer et al. 1991). L-propionyl carnitine has been shown to protect β -thalassaemic erythrocytes and sickle cell anaemia erythrocytes from oxidative stress. β -Thalassaemia is a condition of increased susceptibility to autooxidation caused by multiple factors, for example iron, leading to lipid peroxidation and to damage of membrane proteins. The suggested protective behavior in β -thalassaemia is the binding of L-propionyl carnitine to protein and/or phospholipid domains of the damaged membrane protecting and stabilizing it, hence reducing cell lysis (Palmieri et al. 1994). Similarly, when sickle cell anaemia erythrocytes were treated with hydrogen peroxide a dose-dependent decrease of TBARS formation has been found in the presence of L-propionyl carnitine (Ronca et al. 1994). Just recently, it was demonstrated that L-carnitine supplementation significantly elevated carnitine concentrations in old but not in young

rats (Kalaiselvi and Panneerselvam 1998). Simultaneously urinary carnitine excretion in young rats was increased, suggesting carnitine loss with aging. In addition, it has been shown that antioxidant status, measured by vitamin C and E, catalase, SOD and GPx improved with carnitine supplementation and was highly significant in aged rats. It was suggested that besides radical scavenging activity and recycling of oxidized glutathione, carnitine supplementation might spare ascorbic acid, as ascorbic acid is a cofactor in carnitine biosynthesis.

Carnitine also got attention in the area of exercise performance and weight loss. It was hypothesized that carnitine supplements could improve exercise performance by increasing fatty acid oxidation, thus making more ATP available. Brass reviewed several studies investigating the relationship of carnitine supplementation and exercise performance and concluded that there was no definitive positive correlation between carnitine supplementation and exercise performance (Brass 2000). However, a combination of carnitine, choline and caffeine has been found to increase exercise endurance and a reduction in body fat mass in rats (Hongu and Sachan 2000).

Another aspect of carnitine is its role in alcohol metabolism. Chronic ethanol feeding causes lipid accumulation in liver, which was significantly improved when carnitine was supplemented but not by its precursors lysine and methionine (Sachan et al. 1984). Blood ethanol concentrations were significantly higher in rats supplemented with carnitine compared to controls given equivalent dosages (g per kg body weight) of ethanol (Berger and Sachan 1986). A follow-up study revealed that ethanol metabolism was altered but the absorption of ethanol was unaffected by carnitine (Berger and Sachan 1991). Similarly, in broilers fed carnitine the half-life of ethanol was significantly

prolonged (Smith et al. 1994). In vitro studies have shown that these alterations were related to acetylcarnitine. The presence of acetylcarnitine has been shown to inhibit alcohol dehydrogenase in vitro as it competes with NAD^+ for binding to ADH (Sachan and Cha 1994). Additionally, the metabolism of L-carnitine has been shown to reduce ethanol metabolism after an initial delay. It is thought that L-carnitine must be metabolized to acetylcarnitine first to modulate ethanol metabolism (Cha and Sachan 1995).

II.6.2 Choline

To date choline is classified as a vitamin-like substance found in many foods. Although humans are able to endogenously synthesize choline, some researchers believe that a choline deficient diet would result in choline specific deficiency symptoms. It has been demonstrated, that if choline alone is deficient hepatocarcinomas develop without any other known carcinogens present and the sensitivity to carcinogens increases (quoted by Zeisel 1998). Preneoplastic lesions and 8-hydroxydeoxyguanosine in rat liver DNA were both significantly increased after a choline deficient diet (Nakae et al. 1990). Early signs for choline deficiency are oxidized lipids, proteins and DNA and it is suggested that these are involved in the rapid development of preneoplastic lesions (Zeisel and Blusztajn 1994). A possible reason could be a significant increase of hydrogen peroxide in mitochondria and an impaired respiratory function when choline is deficient (Hensley et al. 2000). The latter could be caused by a decrease in tissue carnitine concentrations and ensuing reduction of fatty acid oxidation, thus affecting respiratory function (Daily et al. 1998). In contrast, choline supplementation resulted in reduced urinary carnitine

excretion, possibly due to enhanced renal reabsorption, and serum carnitine levels were maintained (Dodson and Sachan 1996). In guinea pigs, carnitine concentration in skeletal muscle was higher with choline supplementation and body fat was reduced (Daily et al. 1998). The latter is thought to be related to an increase in fatty acid oxidation.

Choline plays an essential role in cell metabolism because it is closely interrelated with the metabolism of methionine, homocysteine and folate. A deficiency of any of the four compounds has significant effects on the others. Most choline is found in the form of phospholipids such as phosphatidylcholine and sphingomyelin. Other forms of choline found in biological systems are acetylcholine, betaine, platelet-activating factor, choline plasmalogens, phosphocholine, lysophosphatidylcholine and glycerophosphocholine.

Phosphatidylcholine is a substantial compound of cell membranes and is an essential component of very low-density lipoproteins (Yao and Vance 1988). In the case of choline deficiency, very low-density lipoproteins are not produced and triglycerides cannot be transported out of the liver. The triglycerides will accumulate and lead to fatty liver. Choline phospholipids such as sphingomyelin have been shown to be important in signal transduction; their breakdown products can amplify external signals or terminate them by generating inhibitory second messengers as reviewed by Zeisel (1998).

Phosphatidylcholine provides choline for the synthesis of neurotransmitter acetylcholine. Cholinergic neurons in the brain are needed for learning and memory. Acetylcholine is released into the synaptic cleft and acts on the postsynaptic receptors. Learning and memory has been shown to improve when choline was supplemented during brain development, as summarized by Zeisel and Blusztajn (1994). In an in vitro study, it was demonstrated that acetyl-L-carnitine is a precursor for acetylcholine (White

and Scates 1990). Additionally, acetylcarnitine supplementation has been shown to reverse age-related decline in mitochondrial RNA of heart and brain (Gadaleta et al. 1990) and choline uptake in some brain regions (Curti et al. 1989). Another study reported increased choline-acetyltransferase activity in the central nervous system, as an index of cholinergic activity, after acetylcarnitine supplementation (Poivesan et al. 1994).

Acetylcholine is also involved at the neuromuscular junction responsible for the transmission of contraction-generating impulse in muscle. Observations in marathon runners have been shown a 40% reduction in plasma choline levels after running, which may lower acetylcholine release and thereby affect endurance (Conlay et al. 1992). Since acetylcarnitines may serve as acetyl moiety reserve, choline plus carnitine supplement may be beneficial to an endurance athlete (Sachan and Hongu 2000).

II.6.3 Caffeine

Caffeine, a methylxanthine, is the most widely used drug in the world as it is consumed in form of coffee, tea, cocoa and caffeinated soft drinks. It is a psychoactive substance, which is rapidly absorbed. As a lot of research with caffeine has been done in the last few decades it was found that caffeine affects almost every area of physiology, but only if used in pharmacological doses. James (1991) and Spiller (1998) provide in their books summaries of caffeine and its physiological and pharmacological effects.

Besides these actions, a great deal of research has been focused on caffeine as an ergogenic aid. However, there is no clear evidence that caffeine can improve muscular strength, but a great amount of studies detected some ergogenic effect on endurance performance (Lamarine 1998). The endurance performance of cyclists was increased at a

caffeine dose possible to be ingested by drinking coffee. Free fatty acids and glycerol were increased in plasma compared to the control group. Higher caffeine dosages were equally effective but produced higher urinary caffeine levels (Pasman et al. 1995). A reason for this discrepancy might be attributable to caffeine's psycho-stimulatory effect, improving motivation during the exercise to work longer and harder (quoted from Lamarine 1998).

Caffeine has been shown to reduce the threshold of calcium release, thus increasing the calcium efflux from sarcoplasmic reticulum in skeletal muscle cells and inhibiting the reuptake of calcium (Fabiato and Fabiato 1977). In addition, caffeine has been shown to subdue cAMP phosphodiesterase activity hence blocking the breakdown of cAMP. Increased cAMP levels lead to an increase in fatty acid metabolism and subsequently ATP production. However, none of these conditions is possible in vivo, as the levels of caffeine needed to exhibit these mechanisms would be lethal. The most promising explanation for the action of caffeine is its effect on peripheral adenosine receptors. When adenosine binds to its adenosine receptors, it inhibits lipolysis by suppressing adenylate cyclase activity. Chronic caffeine administration has been shown to stimulate adenylate cyclase activity in platelet membranes and suggests that cAMP increases (Zhang and Wells 1990). Additionally, the number of receptors in fat cells was increased, without inhibiting A₁ adenosine receptor mediated lipolysis. Thus, chronic caffeine consumption causes alterations in some peripheral cells. In a human study, increased caffeine administration has been shown to delay glycogen depletion and fatigue during exercise and to increase endurance performance (Pasman et al. 1995). It was suggested that is caused by an increase in lipolysis, however, the mechanisms are unclear.

In recent years, it has been shown that caffeine has a protective effect on radiation damage. Rat mitochondria was exposed to γ -irradiation with and without caffeine and incubated under oxic or anoxic conditions. The presence of caffeine significantly inhibited the formation of TBARS, lipid hydroperoxides and protein carbonyls when exposed to radiation. When incubated with oxygen after radiation all three indices of oxidative stress were significantly reduced. Additionally, glutathione levels and superoxide dismutase were diminished due to radiation but in the presence of caffeine these biomarkers were protected (Kamat et al. 2000).

II.6.4 Dietary Fats

Lipids are an integral part of the human diet delivering essential fatty acids and provide a good source of energy. Animal products and plant oils are the greatest contributors of lipids in the diet. Dietary fatty acids are immediately used for ATP production or stored in cells for later use. If the demand for fatty acids is lower than their availability, they become stored in fat cells. Besides as an energy source, an important role of fatty acids is that of building blocks of membranes. They help to maintain the integrity of the membrane structure of cells and cell compartments. On the other hand, a high dietary fat intake is related to a broad range of diet related diseases such as atherosclerosis, diabetes and cancer. A recent study comparing low and high fat diets found a significant elevation of oxidative damage with the latter (Loft et al. 1998).

Not only the amount, but also the origin of the fats is known to be of importance. The fatty acid composition of the membrane reflects the fatty acid composition of the dietary fat. Thus, the fatty acid composition influences the cell membrane integrity and

survival of the cell. The more polyunsaturated fatty acids are in the dietary fat the greater is the degree of unsaturation in the membrane. A higher degree of unsaturation increases the membrane perceptibility to oxidative damage and thus the formation of lipid peroxidation products (Alexander-North et al. 1994). In addition, the type of polyunsaturated fatty acid used, influences the amount of lipid peroxidation induced by iron (North et al. 1994). High levels of lipid peroxidation can disrupt cell membrane fluidity and then can lead to the oxidation of other cell compounds, cell damage and even cell death.

A comparison of unsaturated and saturated fatty acids has been shown that a diet high in saturated fatty acids protected rat liver from alcohol-induced damage (Cha and Sachan 1994). Although ethanol absorption and alcohol dehydrogenase activity did not differ, blood alcohol clearance was slower in rats fed saturated fatty acids. The results indicate that saturated fatty acids retard ethanol metabolism, which might help reduce ethanol-metabolites and related liver damage.

Different dietary fats and their influence on diseases has been investigated for a long time, but it is only within the last three decades that it has become more evident that lipid soluble antioxidants in oils influence the lipid peroxidation potential of fats (Scaccini et al. 1992). Several oils were studied under various conditions and it became apparent that it is not possible to simply classify oils as bad or good. Depending on the other phyto-compounds in an oil, it might have more beneficial or more undesirable effects on health and disease. **Table 2.4** shows the fatty acid composition of corn oil, fish oil (menhaden oil), palm oil and safflower oil as these oils are used in one of the studies.

Table 2.4Average percentages of fatty acids for selected oils¹

	Dietary fats			
	Corn oil %	Menhaden oil %	Palm oil %	Safflower oil %
Palmitic acid (16:0)	11	19	44	6
Oleic acid (18:1)	24	15	37	12
Linoleic acid (18:2)	58	2	9	74
other PUFA	0	28	trace	Trace

¹ Values are expected means according to Shils et al. 1998

PUFA = polyunsaturated fatty acids

II.6.4.1 Corn Oil

The influence of dietary lipids on changes in the cell membrane and overall cellular antioxidant status is known to be affected by the type of fatty acid.

Polyunsaturated fatty acids are often separated by the location of the first double bond.

They are grouped into n-6 (ω -6) or n-3 (ω -3) polyunsaturated fatty acids depending on the location of the first double bond. Corn oil is a common vegetable oil with the major fraction being linoleic acid, a n-6 polyunsaturated fatty acid. Corn oil is often used in studies because of its polyunsaturated fraction to compare it with predominantly monounsaturated or saturated fatty acids; it is known that polyunsaturated fatty acids are more prone to lipid peroxidation. On the other hand, there is also some evidence that

polyunsaturated fatty acids reduce plasma cholesterol levels (Eritsland 2000). Thus, it is not possible to simply recommend a fat without considering several aspects.

A couple of studies tested a variety of animal fats with corn oil, with and without cholesterol. The goal was to establish in what way the fatty acid saturation influences oxidative damage in rabbits and what role cholesterol plays in it. Compared to a low fat diet all dietary fats increased conjugated dienes and TBARS, and at the same time reducing antioxidant enzymes. The investigators found that corn oil had significant effects on all markers tested and that cholesterol limited some of these effects *in vivo*. Additionally, linoleic acid concentration was highest in liver of corn oil fed rabbits and was prone to higher susceptibility towards oxidants (Slim et al. 1996). A follow-up study has shown the same tendency in aortas with significantly decreased vitamin E levels in the corn oil fed rabbits (Toborek et al. 1997).

II.6.4.2 Fish Oil

The n-6 fatty acids are found in a variety of plant oils but n-3 fatty acids of significant amount are only found in marine fish. In the last three decades fish oil has gotten a lot of attention with respect to risk reduction of diet related diseases such as atherosclerosis or cancer. Eicosanoid biosynthesis needs n-3 or n-6 fatty acids as backbones and both types compete for the same pathway. Although they are homologues, n-3 derived eicosanoids such as PGE₃ have less activity and potency than PGE₂ derived from arachidonic acid (n-6). An increase of n-3 is thought to have an anti-inflammatory and anti-thrombotic effect, however at this time, study outcomes are inconsistent (Jones and Kubow 1998). Inflammatory leukocytes of rats fed either corn or fish oil reflected the

fatty acid composition of the oil and PGE₂ concentration was significantly decreased in the fish oil fed group (Carbonell et al. 1997). Although MDA levels were significantly increased, they found an increased total antioxidant capacity and no reduction in cell number or viability. The authors thought it might have been caused by a reduction in superoxide production because of the lowered PGE₂ levels. In another study, when male rats were fed different oils, the fish oil group was shown to have significantly higher TBARS concentration than the other groups. There were tissue specific differences of the incorporation of fatty acids and thus susceptibility to lipid peroxidation, which was reflected by tissue and urinary TBARS (L'Abbe et al. 1991). Additionally, a higher rate and greater lipid peroxidation was seen when erythrocytes of fish oil fed rats were exposed to oxidants (Garrido et al. 1989). Likewise, plasma of men who ate fish-oil fortified sunflower oil margarine had a significantly reduced lag time of oxidation compared to the group eating pure sunflower oil margarine (Sørensen et al. 1998). The fish oil group had a lower α -tocopherol content in LDL and the total cholesterol increased without changing LDL cholesterol.

II.6.4.3 Palm Oil

Saturated fatty acids have been found to be a predisposing factor to hypercholesterolemia and atherosclerosis. In the late 1980's, palm oil was assumed to be a hypercholesteremic oil because it contains 50% saturated fatty acids (see **Table 2.4**). However, there was some evidence that palm oil lowers cholesterol and slows clotting time; therefore, it would have a small influence on thrombosis formation (Gurr 1991).

Among the vegetable oils, unrefined palm oil is rich in carotenoids and contains about 1000 ppm vitamin E, with substantial amounts of tocopherols and tocotrienols (Nesaretnam et al. 1993). Crude palm oil has an unpleasant flavor, which could be minimized by mixing it with other vegetable oils. It is likely that this would make it more acceptable by consumers as an additional source of β -carotene to reduce vitamin A deficiency in India (Rao 1992).

Refining the oil removes the most of the carotenoids, but not the vitamin E components. Therefore, when rats were fed diets containing either crude or refined palm oil in combination with a high cholesterol or a cholesterol free diet their total serum cholesterol levels were not affected by the palm oil types (Manorama and Rukmini 1992). Other studies have shown contradictory results regarding the influence of palm oil on total plasma cholesterol or plasma lipids, but in a long term feeding study of rats no negative effect on growth or plasma cholesterol was found compared to peanut oil (Hariharan et al. 1992). When hamsters were fed palm oil, significantly lower plasma cholesterol and LDL levels were detected compared to the controls. Similar results were found for the groups fed a palm oil rich triglyceride fraction (Teik and Tan 1992). In a study with normocholesterolemic men receiving 50% of their daily fat through goods baked with palm oil, hydrogenated soybean oil or coconut oil, a tendency towards increasing plasma cholesterol with coconut oil but not with palm oil was observed (Heber et al. 1992).

A comparison of palm oil and vitamin E free palm oil showed that without the vitamin E fraction the number of mammary tumors increased in rats treated with DMBA. (Nesaretnam et al. 1992). A second experiment was conducted where rats were fed diets

containing 20% fat as corn oil with different levels of the tocotrienol rich fraction or palm oil. The results indicated a tendency of lower tumor numbers in rats fed palm oil or corn oil plus 1000 ppm tocotrienol rich fraction (Nesaretnam et al. 1992). In another study, rats were treated the same way but received soybean oil, hydrogenated soybean oil or palm oil as fat source at 5 or 20% levels. The results indicated that palm oil not only decreased the size of tumors but also the number compared to both soybean oil groups (Kritchevsky et al 1992). How palm oil or its derivatives are beneficial to control diseases like atherosclerosis, myocardial injury or certain type of cancer is not completely understood.

Other investigators studied the ability of palm oil and its vitamin E fraction to modulate oxidative damage. When rats were fed a diet supplemented with vitamin E of palm oil, the isolated perfused Langendorff heart was better protected against reperfusion injury (Serbinova et al. 1992). Endogenous lipid peroxidation products did not increase compared to the control. In an in vitro study, tocotrienols possessed a higher antioxidant potency compared to tocopherols. The results suggest that this is part due to a more uniform distribution in the membrane bilayers (Serbinova et al. 1991). After rats were fed palm oil, corn oil or corn oil enriched with vitamin E from palm oil, their liver mitochondria and microsomes were exposed to oxidants. The palm oil group has been shown to have significantly lower lipid peroxidation measured by TBARS compared to the other two groups (Nesaretnam et al. 1993). A time course showed a lower rate and less accumulation of TBARS in the palm oil group. The fatty acid profile with 50% saturated fatty acids in the palm oil could be the reason by reducing the substrate availability for lipid peroxidation. Additionally, the corn oil-vitamin E group had an

intermediate rate but ended with a similar total accumulation of TBARS as the corn oil group, suggesting a combined effect of the degree of saturation and availability of antioxidants. Kamat and Devasagayam (1995) found that 5 μ M of the palm oil tocotrienol-rich fraction was able to significantly inhibit lipid peroxidation and protein oxidation in brain mitochondria. The potency of the tocotrienol-rich fraction to reduce oxidative damage was greater than that of α -tocopherol and was related to the unsaturated side chain of tocotrienols.

II.6.4.4 Safflower Oil

Safflower oil is an unsaturated oil with n-6 fatty acids as approximately 75% linoleic acid (**Table 2.4**). In studies, it is used just as corn oil to compare unsaturated with saturated or n-6 versus n-3 fatty acids. It is possible to find safflower oil in the grocery store but it is more expensive than corn oil. When choosing a vegetable oil, it is not only of interest what nutrients are absorbed, but also if it is possible to influence metabolic pathways in a positive way. One approach is that dietary fatty acids influence fat metabolism by decreasing hepatic triglyceride secretion and increasing hepatic β -oxidation. When a safflower oil diet was compared to fish oil and palm oil diets, a reduction of plasma triglycerides has been found with safflower oil (Halminski et al. 1991). The decrease in the fish oil fed group was greater than in the safflower oil fed group and it was suggested that the position of the double bond might also be important. Interestingly it was found that differences in peroxisomal β -oxidation due to the diet visible after 10 days were diminished after one month and all groups had similar

activities. In another study, a fish oil diet was compared with a safflower oil diet. In both groups, erythrocyte TBARS production significantly increased after the dietary intervention. It was suggested that the overall fat intake, which was about 30 %, might be of greater importance than the type of fatty acid used (Mills et al. 1995).

II.6.4.5 Cis and Trans Fatty Acids

Generally, polyunsaturated fatty acids are found in the cis-form but partial hydrogenation, a common procedure in the food industry, can change up to 50% cis-double bonds into the trans form (Ohlrogge 1983). Another source of trans-fatty acids, although only a small one, are fats from ruminants such as beef fat and dairy products due to microbial hydrogenation (Emken 1983). Trans fatty acids from the diet are found in every tissue, but with a higher accumulation in adipose tissue and are preferably incorporated into triglycerides (Ohlrogge 1983). Knowing that trans fatty acids are absorbed, it was of interest to evaluate the impact of high intakes of hydrogenated oil on biological function. A trans fatty acid free diet reduced tissue levels to negligible amounts within 8 weeks, which indicates that trans fatty acids are metabolized like other fatty acids (Moore et al. 1990). Long term feeding studies with rats did not give any evidence that trans fatty acids have an impact on mortality, cholesterol, growth or life span (Alfin-Slater et al. 1957). Several studies comparing trans fatty acids with saturated or cis fatty acids could not find specific adverse effects of trans fatty acids (Gottenbos 1983). In contrast, trans fatty acids have shown to significantly increase ethanol clearance compared with cis fatty acids or saturated fatty acids in rats (Mong 1998). However, an increase in urinary ethanol excretion might also be responsible for lower blood ethanol

concentrations. A search of the literature revealed that there is no data on the influence of trans fatty acids on oxidative stress.

II.6.5 Exercise

Exercise is known to have many beneficial effects, but there is clear evidence that free radical production increases at the same time. During physical exercise, oxygen consumption increases and more ROS are produced. Ji (1996) stated that exercise not only led to sudden build up of reactive oxygen species but that it was shown in skeletal muscle that exercise training helped to increase general activity and basal level of certain antioxidant enzymes. This was suggested to help cope with increased oxidative stress and protect tissue from excessive oxidative damage. Regular swimming exercise over some weeks did not significantly increase TBARS in rat muscle but instead reduced protein carbonyls and 8-hydroxydeoxyguansine and also induced DT-diaphorase (quinone-reductase). This could be explained by either a better clearance of damaged molecules or a reduction in oxidative stress. The increased DT-diaphorase activity might explain a reduced cancer incidence reported in epidemiological studies (Radák et al. 1999). Regular exercise has been shown to reduce plasma antioxidant concentrations and to increase mitochondrial concentration of the antioxidants retinol, ubiquinone and vitamin E (Quiles et al. 1998, Quiles et al. 1999). Similarly, endurance training in men and women up-regulated certain antioxidant enzymes improving resistance to ROS, which could be the reason for no significant changes in urinary 8-hydroxydeoxyguanosine (Pilger et al. 1997). It has been shown that endurance training up-regulated antioxidant enzymes in rats (Oh-Ishi et al. 1997). Additionally, a bout of acute exercise subsequently

increased superoxide production by neutrophils, which was higher in untrained rats compared to trained rats, suggesting a protective effect of exercise training.

It has been shown that adenosine and R-phenyl isopropyl adenosine (R-PIA), an adenosine agonist, have the ability to stimulate cellular antioxidant enzymes by binding to A₃ adenosine receptor on the cell surface (Maggirwar, et al. 1994). Recently it has been shown that A₃ adenosine receptors in the heart were equally activated by acute exercise or by R-PIA. The addition of theophylline, an antagonist of the A₃ adenosine receptor, diminished the activation of the antioxidant enzymes by exercise or by R-PIA and increased lipid peroxidation. The results suggest that both stimuli use the same activation pathway, as the same antagonist was able to block the enzyme activation (Somani et al. 1997).

Thus, there is good evidence that regular exercise training promotes systems that protect cells against oxidative stress, however, acute or exhausting exercise can increase ROS and subject cells to excessive oxidative damage. After exhausting exercise, rat skeletal muscle showed a significant increase in TBARS and a reduction in membrane fluidity (Li et al. 1999). Nevertheless, trained rats compared to sedentary rats returned to baseline at a faster rate. In other words, untrained rats are more compromised by an acute exercise bout than trained rats. When rats were exercised to exhaustion for two consecutive days and killed immediately after the second term of exercise, both skeletal muscle and liver, had significant stronger free radical signals and increased lipid peroxidation levels compared to the non-exercised rats (Davies et al, 1982). Protein carbonyl formation has also been shown to increase in rat muscle after acute exercise (Reznick et al. 1992).

II.6.6 Alcohol

Although several short-chain alcohols are extensively used in industrial processes, most studies done have been using ethanol simply because it is found in alcoholic beverages. Any amount of alcohol, chronic or single administration, in small or large amounts has an immediate effect. This review will address ethanol-induced alterations of biochemical indices and metabolic pathways, especially fatty acid metabolism.

Ethanol is readily absorbed, carried by blood and readily distributed into every tissue of the body. The products of alcohol metabolism exhibit metabolic and cytotoxic effects in liver, the major alcohol-metabolizing organ. Alcohol dehydrogenase (ADH) in the cytoplasm and the microsomal ethanol oxidizing system (MEOS) in the endoplasmic reticulum account for the greatest amount of ethanol oxidation. ADH oxidizes ethanol to acetaldehyde requiring NAD^+ . During acute ethanol intoxication, this pathway is easily saturated and may be inhibited due to the depletion of NAD^+ (Groff et al. 1995). The concurrent accumulation of ethanol activates MEOS, which have cytochrome P450 units and require oxygen plus NADPH instead of NAD^+ . Thus, MEOS can metabolize large amounts of ethanol without being inhibited by increased NADH. As ethanol is able to induce the biosynthesis of MEOS, it will be constantly up regulated with chronic ethanol use. The increased MEOS capacity can metabolize ethanol more rapidly and establish a state of metabolic tolerance (Groff et al. 1995). This might lead to the ability to ingest larger amounts before signs of intoxication appear.

The biggest problem of large amounts of ethanol derives from the depletion of NAD^+ , which is not only needed for ADH, but also for acetaldehyde metabolism. Aldehyde dehydrogenase has been shown to be diminished with high concentration of

acetaldehyde, but recovered with addition of NAD^+ (Hasumura et al. 1976). Both pathways, ADH and MEOS produce acetaldehyde, which is very reactive and must be oxidized to acetate. Otherwise, acetaldehyde will form covalent bonds with proteins causing enzyme inactivation, GSH depletion and other protein damages (Lieber 1988).

Besides these effects, it is well established that ethanol exposure increases ROS and lipid peroxidation; both are associated with cytochrome P450 activity. It is suggested that besides hydrogen peroxide, cytochrome P450 may also directly form hydroxy-alcohol radicals. Of special concern in this reaction is cytochrome P4502E1, an ethanol inducible form (Albano et al. 1991, Lieber 1997). Reviews state that cytochrome P450, ROS, hydroxy-alcohol radicals and cell loss were all found to be increased during chronic alcohol exposure and were diminished when cytochrome P450 inhibitors were added (Baker and Kramer 1999, Colton and Zakhari 1997). The enhanced production of ROS during ethanol and acetaldehyde metabolism and depletion of GSH may amplify lipid peroxidation (Lieber 1988). Hence, it is likely that oxidative stress is partially involved in alcohol induced cytotoxicity.

The shift in the NAD^+/NADH ratio towards NADH not only leads to acetaldehyde accumulation, but also limits other NAD^+ dependent dehydrogenases in their reactions. This may result in a shift of lactate dehydrogenase to produce lactate instead of pyruvate and may increase glycerol 3-phosphate and malonyl CoA, both contributing to increased fatty acid synthesis (Groff et al. 1995). At the same time, acute ethanol feeding decreases the activity of carnitine-palmitoyltransferase-I (CPT-I) and the rate of fatty acid oxidation (Guzman and Geelen 1988). In addition, long-term ethanol feeding was shown to increase the sensitivity of CPT-I to malonyl CoA, which inhibits

CPT-I activity at even lower concentrations than under normal conditions (Guzman and Geelen 1988). These factors have been suggested to be the reason for the development of fatty liver. Reduced fatty acid oxidation may also be responsible for altered plasma carnitine concentrations in alcohol-induced cirrhosis. Studies in cirrhotic patients have shown that plasma carnitine and especially long-chain acylcarnitines were elevated (Kraehenbuehl and Reichen 1997, Amodio et al. 1990, Fuller and Hoppel 1988). The transfer of acyl groups to carnitine could be an attempt to increase free CoA and reactivate fatty acid oxidation (Fuller and Hoppel 1988). Increased plasma acylcarnitines levels may also be due to accelerated skeletal muscle protein turnover and subsequent enhanced carnitine biosynthesis (Kraehenbuehl and Reichen 1997).

The nonoxidative metabolism of alcohols refers for example to the transphosphatidylation of alcohols with phosphatidyl moieties to form phosphatidylalcohol. Phospholipase D catalyzes the biosynthesis of phosphatidic acids, which are essential for intracellular signaling and protein kinase activation, but if short-chain alcohols such as ethanol are present, phospholipase D transfers the phosphatidyl moieties onto the alcohol instead onto water (Seidler et al. 1996). Baker and Kramer (1999) suggested that the decrease in phosphatidic acids might then affect normal cell function as it is an activator for specific protein kinases and can induce cell proliferation. On the other hand, phosphatidylethanol has been shown to have a direct effect on Ca^{2+} -ATPase activity in human erythrocytes (Suju et al. 1996). Stimulation with calmodulin or acidic phospholipids was augmented with the addition of phosphatidylethanol. In addition, the affinity of the enzyme to Ca^{2+} and the maximum velocity were increased.

Chapter III

Materials and Methods

III.1 Experimental Design and Treatment of Animals

This chapter contains only a brief description of the experimental animals, diet and sample collections, because the original studies were carried out by others in Dr. Sachan's laboratory. The University of Tennessee Institutional Review Board for the use of laboratory animals approved the protocols of all studies.

III.1.1 Choline, Carnitine and Caffeine Study

Twenty 7-week old male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were studied for five weeks. The rats were individually housed under conditions controlled for light (12-hour light-dark cycle), temperature (20-22 °C) and relative humidity (50%). The rats were given free access to diet and water. The endogenous concentrations of choline and carnitine in the commercial diet, Teklad 22/5, were 2.1 g and 30 mg per kg diet, respectively. The supplemented diet was commercial chow fortified with caffeine, carnitine, and choline (CCC) at the rate of 0.1, 5 and 11.5 g per kg non-purified diet, respectively.

For the first week, all rats consumed the non-supplemented diet. In the second week, the rats were randomly divided into two groups and assigned to the supplemented or non-supplemented diet (n = 10). At week three, five rats of each dietary group were

randomly selected for exercise and were run on a treadmill five days a week. The diet and exercise regimens were continued in weeks four and five.

At the end of the fifth week, the rats were anesthetized and killed; blood samples were collected and immediately centrifuged at $2000 \times g$ for 10 min. The serum was stored at -80°C until analyzed for various metabolites. Following blood collection, liver, heart, kidney, skeletal muscle and brain were excised from the carcasses, rinsed with saline solution, blotted dry and frozen in liquid nitrogen and stored at -80°C . A detailed description of the study can be found in the original article (Hongu and Sachan 2000) and Hongu's dissertation (in press).

III.1.2 Different Dietary Fats Study

Twenty-four male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were studied for eight weeks. The rats were housed under the same conditions as described in the first study. After the animals were acclimatized for one week, they were randomly divided into four groups ($n=6$). During the eight weeks of the experiment, the rats had free access to water and diet. The diet was a modified AIN⁷⁶ diet containing 10% corn oil, safflower oil (Sigma Chemical, St. Louis, MO), fish oil (Sigma Chemical, St. Louis, MO) or palm oil (Gift of the Palm Oil Research Institute of Malaysia) as the only source of fat.

On day 60, the rats were given an oral dose of 3 g ethanol per kg body weight in a 13% aqueous solution (v/v). A second dose of ethanol was given two days later and after 24 hours all rats were anesthetized with metoxyflurane (Pitman-Moore, Inc., Mundelein, IL) and cardiac blood was collected in heparin-containing tubes. Blood samples were

immediately centrifuged and the serum stored at -80°C until analyzed for metabolites. The rats were killed by exsanguination and their livers perfused with saline solution, removed, blotted dry, frozen in liquid nitrogen and immediately stored at -80°C .

III.1.3 Cis and Trans Fatty Acids Study

For this study, twenty-four Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were studied for two weeks. After one week of feeding Purina Rodent Chow (Ralston Purina Co., St. Louis, MO), the rats were randomly assigned to one of two dietary groups ($n=12$). The rats were individually housed under conditions controlled for light (12-hour light-dark cycle) and temperature ($23 \pm 2^{\circ}\text{C}$). During the experiment, the rats had free access to diet and water; they were fed a modified AIN⁷⁶ diet with 10% (w/w) fat containing predominantly cis (CFA) or trans (TFA) fatty acid (Appendix 1).

At the end of experimental period, six rats of each group were given an oral dose of 3 g ethanol per kg body weight as 13% aqueous solution (v/v) 2 hours before all animals were killed. The rats were anesthetized with Metaphane and cardiac blood samples were collected. Blood samples were immediately centrifuged at $2000 \times g$ for 10 min and the serum stored at -80°C until analyzed. The animals were killed and their livers perfused with normal saline solution, removed from the carcasses, blotted dry, frozen in liquid nitrogen and stored at -80°C . A detailed description is found the doctoral thesis of Mei-Shin Mong (Mong 1999).

III.2 Reagents and Treatment of Samples

Reagents were stored at room temperature or in the refrigerator. Unless mentioned otherwise, they were kept in dark glass bottles and regularly checked for a change in pH, which was readjusted if necessary. All samples were stored at -80°C with a small overhead of air. Before the assays, serum samples or tissue homogenates were briefly thawed in water and placed in ice for complete thawing. Samples of one tissue type were always assayed in sets of minimum four. If a result of single determinations appeared to be a complete outlier, the determination was repeated.

III.2.1 Homogenization of Tissue Samples

Reagents:

1. 0.154 M Potassium chloride buffer (KCl) containing 0.01 M potassium phosphate (monobasic KH_2PO_4) adjusted to pH 7.4 with 4 N NaOH
2. 0.9 % normal saline solution was used for samples of choline, carnitine and caffeine study

Procedure: Approximately 300 mg frozen tissue samples were weighed out. The samples were transferred into a Teflon-glass homogenizer and 500 μl buffer was added. The tissue samples were homogenized with 3-6 strokes, keeping the homogenizer in ice. Afterwards, the homogenates were pipetted into microcentrifuge tubes and the homogenizer washed with another 500 μl buffer; the wash was added to the homogenate. Buffer was added to the tubes to get a homogenate volume of 1.5 ml. The samples were centrifuged at $500 \times g$ at 4°C for 10 min in a Micromax RF microcentrifuge (IEC,

Needham Heights, MA) and the supernatants collected. The supernatants were re-centrifuged at $10.000 \times g$ at 4°C for 10 min, collected into clean microcentrifuge tubes and frozen at -80°C until further use. The pellets were discarded.

III.3 Protein Determination

The protein content of serum and tissue homogenates was determined by the method described by Bradford using a commercial dye reagent (Bradford 1976). The principle of the method is the binding of Coomassie blue with protein and a subsequent shift in the absorption maximum from 465 to 595 nm. The assay is sensitive to changes of $5 \mu\text{g}$ protein per ml.

Reagent:

1. Concentrated commercial dye reagent (BioRad, Hercules, CA), containing Coomassie Brilliant Blue, phosphoric acid and methanol.
2. Bovine serum albumin (BSA) (Fisher Scientific, Fair Lawn, NJ)

Procedure: One part of the concentrated dye was diluted with four parts of distilled water. The solution was filtered through a Whatman #1 filter to remove any particles and then stored in the refrigerator. For the standards, a stock solution of 1 mg protein/ml was prepared by dissolving 5 mg BSA carefully in 5 ml distilled water. Then five standards of concentrations of 0.1 to 1 mg protein/ml were made and stored at -4°C (**Table 3.1**). One serum or tissue homogenate sample was tested in various dilutions; a dilution factor was selected to contain approximately 0.5 mg protein/ ml and was used for all samples of the specific tissue. The diluted samples (25 μl) or the standards were pipetted into different

Table 3.1

Protein standards for protein determination by the Bradford method

Standard	Protein content mg	Stock solution	Distilled water
	mg	μ l	μ l
1	0.1	100	900
2	0.25	250	750
3	0.5	500	500
4	0.75	750	250
5	1.0	1000	-

single-use glass test tubes, while the Bradford solution was used for the blank. The standards and some samples were prepared in duplicate. Bradford solution (1.25 ml) was added to each tube and the mixture immediately vortexed. The tubes were left at room temperature for 15 min and the contents were transferred into disposable plastic cuvettes. Absorbance was read at 595 nm against a blank in a Genesys 5 spectrophotometer (Spectronic, Rochester, NY).

Determination of Protein Concentration: The mean of each duplicated protein standard were used and a standard curve plotted, which was linear in range of 0.1 to 1 mg protein per ml. A typical standard curve is given in **Figure 3-1**. The regression was calculated and the protein concentrations in each sample calculated as follows:

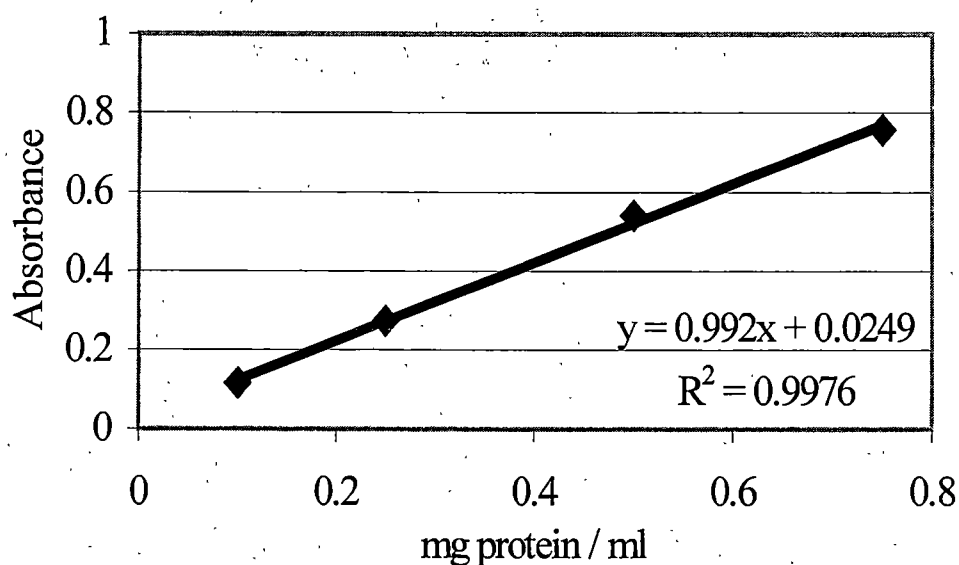


Figure 3-1 Typical standard curve for protein using the Bradford method

mg protein /ml homogenate or serum =

$$[(\text{Abs.} + \text{Slope}) / \text{Standard deviation}] \times \text{DF}$$

Abs. = Absorbance of sample against blank
 DF = dilution factor

III.4 Determination of TBARS

TBARS in serum and tissue homogenates were determined with minor modifications, using the method originally described by Placer et al. (1966) and modified by Ohkawa et al. (1979). The assay is based on the principle of the formation of a pink pigment when one lipid peroxide molecule, such as MDA, reacts with two thiobarbituric

acid molecules at an acidic pH. The development of the pink color can be estimated by the absorbance at 532 nm.

Reagents:

1. 8.1 % Dodecyl sodium sulfate (SDS): (Fisher Scientific Co., Fair Lawn, NJ)
2. 0.8 % Thiobarbituric acid (TBA): After TBA (Sigma-Aldrich, St. Louis, MO) was added, the solution was carefully warmed and about 25 μ l of 10 M NaOH were added if TBA was not completely dissolved.
3. 20 % Acetic acid adjusted to pH 3.5 with 10 M NaOH.
4. 1-Butanol/pyridine solution (v/v, 15/1): The mixture was stored and used under the hood at all times.
5. 1,1,3,3 Tetraethoxypropane (MDA) (Sigma-Aldrich, St. Louis, MO)

Procedure: For the MDA standards a stock solution of 1mM MDA was made by adding 12 μ l MDA to 49.988 ml distilled water. The stock was then used to make two working standards. For working standard A, 500 μ l of the stock solution was diluted in 49.5 ml distilled water. For working standard B, 5 ml of the stock solution were diluted in 45 ml distilled water. The working standards were then used to prepare seven standards (**Table 3.2**), which were immediately added into the test tubes. SDS (0.1 ml) and acetic acid (0.75 ml) were pipetted in 10 ml glass test tubes. Tissue homogenates or serum (100–350 μ l) were added to each tube to have about 1 mg protein in the incubation mixture, while 400 μ l distilled water was used for the blank. TBA solution (0.75 ml) and distilled water were added to get a total volume of 2 ml. The test tubes were carefully vortexed,

Table 3.2

MDA standards for TBA assay

Concentration	Working standard A	Working standard B	Distilled water
nmol/ ml	μ l	μ l	μ l
0.2	20	-	80
0.5	50	-	50
0.8	80	-	20
1.0	100	-	0
2.0	-	20	80
3.0	-	30	70
5.0	-	50	50

covered with a loosely fitting glass stopper and incubated in a Precision tube block heater (GCA corp., Chicago, IL) at 97 °C for 60 minutes. After cooling the tubes in ice water, 0.5 ml distilled water and 2.5 ml butanol/pyridine mixture were added to have a final assay volume of 5 ml. The tubes were shaken vigorously by vortexing and then centrifuged in a Model TJ-6 table centrifuge (Beckman, Waldwick, NJ) at 3050 rpm at 4 °C for 10 min. Before the readings, the tubes were carefully shaken to reverse some phase separation within the organic layer and about 750 μ l from the top organic layer were pipetted into a 1 ml quartz cuvette. The samples were read against the blank at 532

nm after 1 min resting in a Genesys 5 spectrophotometer (Spectronic, Rochester, NY).

The concentrations were calculated as follows:

nmole MDA / mg protein =

$$\{[(V \times \text{Abs.}) / 0.151] \times \text{DF}\} / (\text{mg protein/ml sample})$$

V = final assay volume (5 ml)

Abs. = absorbance at 532 nm

0.151 = molar extinction coefficient for TBARS in pyridine-butanol

DF = dilution factor depending on homogenate volume used for the assay

A typical standard curve is shown in **Figure 3-2**. The relationship between absorbance and the standard was linear in the range of 0.1 to 5 nmol per ml.

Comments: The developed color was found to be stable for at least 7 hours after cooling (Placer et al. 1966). Ohkawa et al. found that the color was stable for at least 3

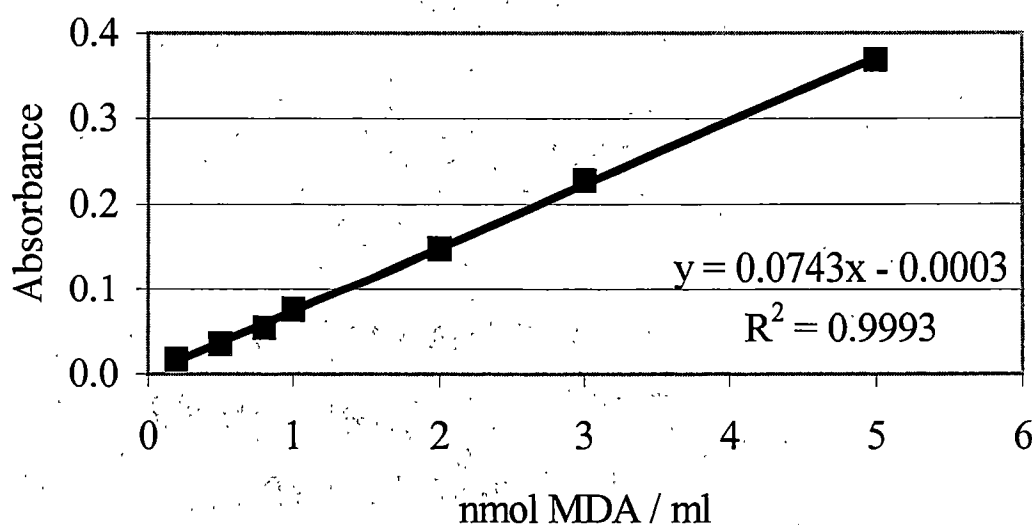


Figure 3-2 Typical standard curve for MDA using the TBA assay

hours once a sample was extracted with butanol/pyridine. In this study, one set of homogenates or serum samples were incubated together. After the samples were cooled, the rest of the assay was done in batches of about 10 samples and each set included a blank. After the solvent was added, the samples were always immediately assayed.

III.5 Determination of Lipid Hydroperoxides

The FOX (ferrous ion oxidation with xylenol orange) assay was used to determine water-soluble lipid hydroperoxides in serum and tissue homogenates as described by Wolff (Wolff 1994). The principle is that hydroperoxides selectively oxidize ferrous to ferric ion in dilute acid. The ferric ions are determined using xylenol orange, a ferric sensitive dye, as an indirect measure of hydroperoxides. The binding of xylenol orange with ferric ion produces a blue-purple complex with an absorbance maximum at 560 nm. Sorbitol is added to the FOX solution to scavenge oxyl radicals produced by a direct reaction between ferric ions and hydroperoxides. Oxyl radicals are converted to hydroperoxides and thus improve the ferric ion yield and the color enhancement. Due to the high extinction coefficient ($2.2 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) at 560 nm hydrogen peroxide concentrations of $2 \mu\text{M}$ can be detected in the test solution (Wolff 1994).

Reagents:

1. 75 mM Sulfuric acid (H_2SO_4)
2. 2.5 mM Ammonium ferrous sulfate solution: The ammonium ferrous sulfate crystals were immediately dissolved in 75 mM sulfuric acid to avoid autooxidation at physiological pH.
3. 1 M Sorbitol solution (Sigma-Aldrich, St. Louis, MO)

4. 1 mM Xylenol orange solution (Sigma-Aldrich, St. Louis, MO)
5. 25 mM Sulfuric acid (H_2SO_4)
6. 30 % Hydrogen peroxide (Fisher Scientific Co., Fair Lawn, NJ)

Procedure: The solutions were made fresh before each assay. First, the standards were prepared, 515 μl of hydrogen peroxide was added directly into 4485 μl distilled water. Then 10 μl of this solution was added to 9990 μl distilled water to make the stock solution A with a concentration of 100 μM . From the stock solution A 100 μl were added to 900 μl distilled water to make stock solution B with a concentration of 10 μM . The stock solutions were used to prepare six standards (**Table 3.3**). For the FOX solution

Table 3.3

Hydrogen peroxide standards for the FOX assay

Standard	Concentration	A	B	Water	FOX
		μl	μl	μl	μl
1	0.0 μM	-	-	50	950
2	0.25 μM	25	-	25	950
3	0.5 μM	50	-	-	950
4	1.0 μM	-	10	40	950
5	2.5 μM	-	25	25	950
6	5.0 μM	-	50	-	950

30 ml sorbitol solution, 30 ml xylenol orange solution and 30 ml ammonium ferrous sulfate solution were added with to 210 ml of 25 mM H₂SO₄ and mixed thoroughly. FOX solution (950 µl) was pipetted into 1.5 ml microcentrifuge vials followed by 50 µl serum, tissue homogenate or H₂O₂ standards, while for the blank 1 ml FOX solution was used. The mixture was vortexed and incubated at room temperature for a minimum of 30 min to allow full color development. The standards and samples were transferred into disposable plastic cuvettes and the absorbance read against the blank at 560 nm.

Determination of Lipid Hydroperoxide Concentration: The mean of each duplicated H₂O₂ standard (1 – 5 µM) was used and a standard curve plotted, which was linear in the 0 - 5 µM concentration range. A typical standard curve is given in **Figure 3-3**. The regression was calculated and the hydroperoxide concentration in each sample calculated as follows:

$$\begin{aligned} \text{nmol lipid hydroperoxide / mg protein} = \\ \{[(\text{Abs.} + \text{Slope}) / \text{Standard deviation}] \times 20\} / \\ (\text{mg protein/ ml homogenate or serum}) \end{aligned}$$

Abs. = Absorbance of sample against blank

Comments: If after incubation flocculated material was present, the material had to be removed before the reading by centrifugation. The dilution factor of 20 was necessary to acknowledge the dilution of the undiluted sample by the incubation mixture. To verify that authentic hydroperoxides are present in the samples, 200 units catalase were added to a tube before the sample. Additionally, recovery experiments with H₂O₂ were carried out.

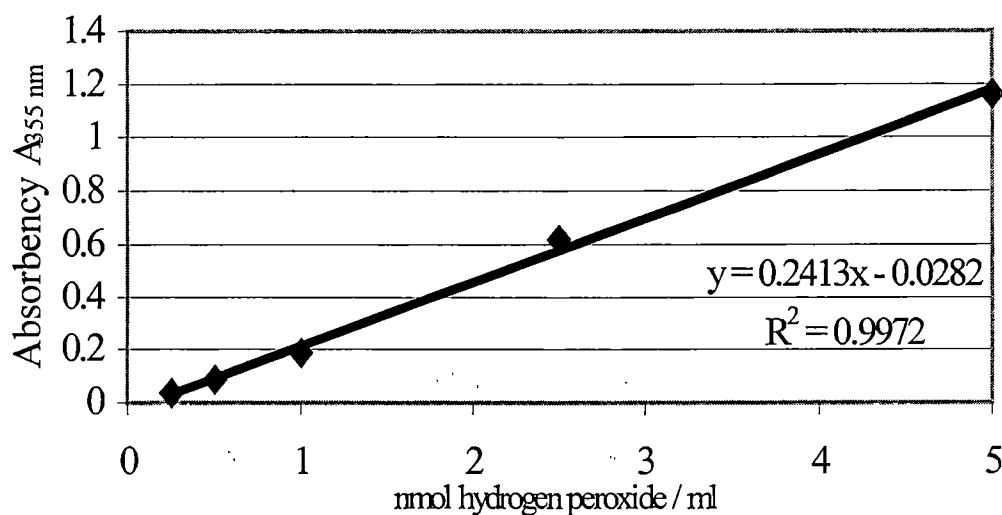


Figure 3-3 Typical standard curve of hydrogen peroxide for the FOX assay

III.6 Determination of Protein Carbonyls

Protein carbonyls in serum and tissue homogenates were determined based on the method described by Levine and colleagues and later modified by Reznick and partner (Levine et al. 1990, Reznick and Packer 1994). The principle of this assay is the reaction of protein carbonyls with 2,4-dinitrophenylhydrazine to form protein hydrazones. Due to technical problems, minor modifications were made. For the assay, a specific homogenization technique and washing steps were required, which together caused an approximate loss of 12 % protein. Thus, the protein content was determined as part of the assay. The assay is sensitive to detect protein carbonyls in samples with only 0.5 mg per

sample and will give reproducible results if the carbonyl content is 0.2 to 1 mg per mg protein.

Reagents:

1. 2.5 M Hydrochloric acid (HCl)
2. 10 mM 2,4 Dinitrophenylhydrazine (DNPH) in 2.5 M HCl
3. Absolute ethanol/ethyl acetate solution (1/1, v/v)
4. 40% Trichloro-acetic acid (TCA) solution (w/v): A stock solution of 40 % TCA was prepared and before the assay the stock solution was used to make two working solutions of 10 % and 20 %, respectively.
5. 50 mM Sodium-potassium phosphate buffer adjusted with 10 N NaOH to pH 7.4
6. 10% Streptomycin sulfate (Sigma-Aldrich, St. Louis, MO) in 50 mM sodium-potassium phosphate buffer (pH 7.4): The solution was stored in the refrigerator.
7. Homogenizing buffer containing 1mM EDTA, 0.1% digitonin and protease inhibitors: Before homogenization, 60 mg digitonin (Fisher Scientific Co., Fair Lawn, NJ) was dissolved in approximately 5 ml sodium-potassium phosphate buffer and 3 ml ethanol. The digitonin mixture and 1.2 ml of 10 mM EDTA were then added to 40 ml sodium-potassium-phosphate buffer, followed by 200 μ l of a protease inhibitor cocktail [containing DMSO as solvent, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64), bestatin, leupeptin and aprotonin but no metal chelators] (P8340, Sigma-Aldrich, St. Louis, MO). The volume was then adjusted to 60 ml using the sodium-potassium phosphate buffer. The slightly milky looking homogenizing buffer was kept on ice during the homogenation procedure.

8. 6 M Guanidine hydrochloride containing 20 mM potassium phosphate adjusted with 10 M HCl to pH 2.3

9. Bovine serum albumin

Procedure: Frozen tissues samples were weighed out on a small plastic dish. The approximate weight of the different tissues were: liver ~80 mg, kidney ~100 mg, brain ~150 mg, testis ~130 mg or skeletal muscle ~130 mg. Homogenizing buffer (1.5 ml) was added immediately and the tissue samples were finely minced with a surgery knife. The samples were incubated at room temperature for 15 min. Afterwards the fluids were carefully poured into a 1.5 ml microcentrifuge tube, keeping solid tissue in the plastic dish. The samples were centrifuged in a Micromax RF microcentrifuge (IEC, Needham Heights, MA) at 6000 x g and 4 °C for 10 min. The debris were removed by transferring the supernatants in new tubes and frozen at -80 °C until the assay was performed. For the protein standards a stock solution with a concentration of 2 mg protein/ml was prepared dissolving 10 mg BSA in 5 ml guanidine hydrochloride solution. Five different standards with concentrations from the concentration range 0.25 to 2 mg protein were prepared and stored at -4°C (Table 3.4). Two 200 µl samples of tissue homogenate or protein standard were pipetted in two 2 ml microcentrifuge tubes. For serum samples, 20 µl sample plus 180 µl distilled water were used, which was due to the higher protein content in serum. To one tube 800 µl of 10mM DNPH in 2.5 M HCl were added, while to the other tube 800 µl of the 2.5 M HCl were added. The tubes were vortexed and incubated in a dark place at room temperature for 1 hour. Every 15 min the samples were vortexed. Afterwards, 1000 µl of 20% TCA solution were added to both DNPH and HCl tubes to

Table 3.4

Protein standards for protein carbonyl assay

Standard	Protein content	Stock solution	Guanidine Hydrochloride
	mg/ml	μ l	μ l
1	0.25	125	875
2	0.5	250	750
3	1.0	500	500
4	1.5	750	250
5	2.0	1000	-

get a final concentration of 10% TCA (w/v). The tubes were left for 10 min on ice and then centrifuged at 10.000 x g and 4 °C for 5 min in the Micromax RF microcentrifuge (IEC, Needham Heights, MA). The supernatants were discarded and 800 μ l of 10% TCA were added to the precipitates. The pellets were mechanically broken with the help of a glass rod. The tubes were vortexed and centrifuged at 10.000 x g and 4 °C for 10 min. The supernatants were discarded and a second wash using 800 μ l ethanol/ ethyl acetate solvent was performed to remove free DNPH and lipids. After breaking the debris with the glass rod and vortexing, the samples were re-centrifuged using the same conditions, discarding the supernatants. The ethanol/ethyl acetate step was repeated twice. To the final precipitates, 1 ml of guanidine hydrochloride solution was added. The samples were vortexed and incubated in a water bath incubator (GCA Corp., Chicago, IL) at 37 °C for

10 min, interrupted every 2.5 min for vortexing; this was done to ensure that everything was dissolved. If any insoluble material was present in a sample, the sample was re-centrifuged for 3 min at 9800 x g.

Determination of Protein Content: The protein standards and samples treated with HCl were read against a blank of guanidine hydrochloride using 1 ml quartz cuvettes at 280 nm in a Genesys 5 spectrophotometer (Spectronic, Rochester, NY). The mean of each duplicated protein standard (0.25 – 2 mg/ml) was used and a standard curve plotted. A typical standard curve was linear in range of 0.25 to 2 mg per ml (**Figure 3-4**). The regression was calculated and the protein concentrations for each sample calculated as follows:

mg protein / ml homogenate or serum =

(Abs. + Slope) / Standard deviation x DF

Abs. = Absorbance of sample against blank

DF = Dilution factor, 5 for tissue homogenates and 50 for serum samples

Determination of Protein Carbonyl Content: To determine the protein carbonyl content all samples treated with DNPH were read against their own double made with HCl at 355 nm. The protein carbonyl concentration in each sample was calculated as follows:

nmol protein carbonyls / mg protein =

$[(\text{Abs } 355 \text{ nm} \times 45.45 \text{ nmol} / 0.2 \text{ ml}) \times \text{DF}] / (\text{mg protein/ml})$

ϵ = Absorption coefficient of 22,000/M = $2.2 \times 10^4 / 10^6$ nmol/ml

DF = Dilution factor, 5 for tissue homogenates and 50 for serum samples

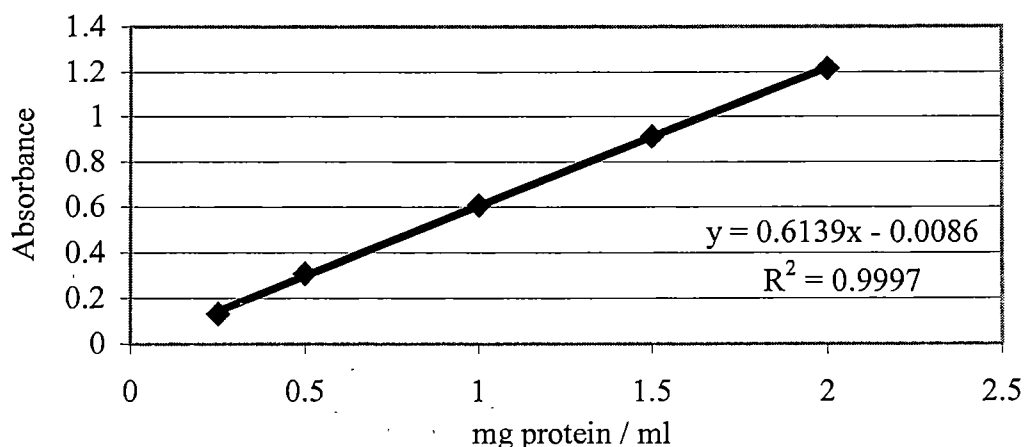


Figure 3-4 Typical standard curve for protein for the carbonyl assay

Comments: First, because of the careful mincing of the tissue samples the protein yield was above 1 mg protein/ml homogenate and thus concentrating of the sample by drying or precipitating with TCA was not necessary. The authors mentioned that because of the careful mincing the reproducibility of the assay was improved. Second, nucleic acids may interfere with correct readings and overestimate carbonyl concentration. To check for interfering nucleic acids the ratio of the absorbencies at 280 and 260 nm were read for a few samples of the same tissue. Whenever the ratio of 280/ 260 nm was less than 1, the nucleic acids had to be removed with 150 μ l of 10% streptomycin solution. The mixture was incubated at room temperature for 15 min and centrifuged at 6000 x g for 10 min. The supernatant was used for the assay. It is expected that because of a careful and gentle mincing this treatment will not be necessary.

III.7 Statistics

All results are presented as group means $\pm$ standard error of mean. Data were analyzed using two-way ANOVA. Duncan's post hoc test was applied for multiple comparisons. The statistical significance level was set at $p \leq 0.05$.

Chapter IV

Results

IV. 1 Effects of Choline, Carnitine and Caffeine

The results of the effects of exercise (NS/E), supplementation (S/NE) and the combination of both (S/E) compared to the control without supplement and exercise (NS/NE) on TBARS, lipid hydroperoxides and protein carbonyls in serum, liver, muscle, brain, testis and kidney are presented in **Table 4.1 to 4.6**. In **Table 4.7**, the correlations between concentrations of TBARS and lipid hydroperoxide or protein carbonyl are summarized for all tissues. There was no correlation between lipid hydroperoxide and protein carbonyl concentrations. The data present in **Table 4.1 to 4.6** are illustrated in **Figure 4-1 to 4-3**.

Serum: The supplemented and exercised groups had significantly higher levels of TBARS ($p \leq 0.01$) compared to the NS/NE group (**Table 4.1**). Protein carbonyl concentrations were significantly higher in both supplemented groups ($p \leq 0.05$), but not in the NS/E group. A significant interaction of supplement and exercise was observed for TBARS ($p \leq 0.01$). TBARS and hydroperoxides were positively correlated, whereas TBARS and protein carbonyls were negatively correlated ($p \leq 0.01$) (**Table 4.7**).

Table 4.1

Comparison of serum TBARS, hydroperoxide and protein carbonyl concentrations in rats fed diets with or without supplement and with or without exercise¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/ mg protein		
NS/NE	0.09 ± 0.01 ^a	0.54 ± 0.03	2.32 ± 0.04 ^a
NS/E	0.20 ± 0.02 ^b	0.39 ± 0.04	2.49 ± 0.10 ^{ab}
S/E	0.23 ± 0.02 ^b	0.42 ± 0.03	2.80 ± 0.10 ^b
S/NE	0.23 ± 0.01 ^b	0.40 ± 0.05	2.81 ± 0.18 ^b
	Statistical significance ²		
Supplement	< 0.001	NS	0.003
Exercise	0.003	NS	NS
S x E ³	0.004	NS	NS

NS = no supplement, S = supplement, NE = no exercise, E = exercise

¹ Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$

² Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)

³ Supplement and exercise interaction

Table 4.2

Comparison of hepatic TBARS, hydroperoxide and protein carbonyl concentrations in rats fed diets with or without supplement and with or without exercise¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/mg protein		
NS/NE	0.42 ± 0.01 ^a	3.55 ± 0.11	5.33 ± 0.24
NS/E	0.40 ± 0.04 ^a	3.84 ± 0.05	5.26 ± 0.16
S/E	0.57 ± 0.03 ^b	3.57 ± 0.13	5.93 ± 0.20
S/NE	0.49 ± 0.03 ^b	3.31 ± 0.20	5.41 ± 0.29
	Statistical significance ²		
Supplement	< 0.001	NS	NS
Exercise	NS	NS	NS
S x E ³	NS	NS	NS

NS = no supplement, S = supplement, NE = no exercise, E = exercise

¹ Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$

² Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)

³ Supplement and exercise interaction

Table 4.3

Comparison of skeletal muscle TBARS, hydroperoxide and protein carbonyl concentrations in rats fed diets with or without supplement and with or without exercise¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/ mg protein		
NS/NE	2.52 ± 0.26	1.05 ± 0.11	8.89 ± 0.27 ^{ab}
NS/E	2.01 ± 0.14	0.94 ± 0.07	7.97 ± 0.13 ^a
S/E	2.49 ± 0.35	1.30 ± 0.25	8.11 ± 0.28 ^{ab}
S/NE	2.64 ± 0.54	0.89 ± 0.05	9.15 ± 0.56 ^b
	Statistical significance ²		
Supplement	NS	NS	NS
Exercise	NS	NS	0.012
S x E ³	NS	NS	NS

NS = no supplement, S = supplement, NE = no exercise, E = exercise

¹ Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$

² Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)

³ Supplement and exercise interaction

Table 4.4

Comparison of brain TBARS, hydroperoxide and protein carbonyl concentrations in rats fed diets with or without supplement and with or without exercise¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/ mg protein		
NS/NE	2.31 ± 0.20	5.52 ± 0.24	9.87 ± 0.50 ^b
NS/E	2.27 ± 0.06	5.56 ± 0.52	7.91 ± 0.48 ^a
S/E	2.38 ± 0.17	5.88 ± 0.30	8.06 ± 0.17 ^a
S/NE	2.55 ± 0.20	4.91 ± 0.56	10.83 ± 0.52 ^b
	Statistical significance ²		
Supplement	NS	NS	NS
Exercise	NS	NS	< 0.001
S x E ³	NS	NS	0.001

NS = no supplement, S = supplement, NE = no exercise, E = exercise

¹ Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$

² Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)

³ Supplement and exercise interaction

Table 4.5

Comparison of testes TBARS, hydroperoxide and protein carbonyl concentrations in rats fed diets with or without supplement and with or without exercise¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/ mg protein		
NS/NE	0.50 ± 0.09 ^b	7.95 ± 0.38	7.17 ± 0.77
NS/E	0.28 ± 0.02 ^a	9.14 ± 0.46	7.13 ± 0.31
S/E	0.61 ± 0.08 ^b	8.33 ± 0.59	8.10 ± 0.44
S/NE	0.64 ± 0.02 ^b	8.83 ± 0.14	8.17 ± 0.32
	Statistical significance ²		
Supplement	0.001	NS	NS
Exercise	NS	NS	NS
S x E ³	NS	NS	NS

NS = no supplement, S = supplement, NE = no exercise, E = exercise

¹ Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$

² Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)

³ Supplement and exercise interaction

Table 4.6

Comparison of kidney TBARS, hydroperoxide and protein carbonyl concentrations in rats fed diets with or without supplement and with or without exercise¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/ mg protein		
NS/NE	3.35 ± 0.30	2.24 ± 0.13	13.55 ± 0.12
NS/E	3.34 ± 0.14	1.97 ± 0.12	13.49 ± 0.42
S/E	3.15 ± 0.25	2.29 ± 0.18	13.66 ± 0.22
S/NE	3.29 ± 0.25	2.23 ± 0.08	12.68 ± 0.19
	Statistical significance ²		
Supplement	NS	NS	NS
Exercise	NS	NS	NS
S x E ³	NS	NS	NS

NS = no supplement, S = supplement, NE = no exercise, E = exercise

¹ Values are means ± SEM (n = 5) and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$

² Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)

³ Supplement and exercise interaction

Table 4.7

Correlation coefficients (R) between TBARS and hydroperoxide or protein carbonyl concentrations in serum and tissues of rats fed diets with or without supplement and with or without exercise

	R	P-value
Serum		
Hydroperoxides	0.626	0.004
Protein carbonyls	-0.575	0.008
Liver		
Hydroperoxides	-0.164	0.502
Protein carbonyls	0.472	0.041
Skeletal muscle		
Hydroperoxides	0.475	0.034
Protein carbonyls	0.155	0.513
Brain		
Hydroperoxides	-0.070	0.768
Protein carbonyls	-0.071	0.765
Testes		
Hydroperoxides	-0.033	0.890
Protein carbonyls	0.030	0.900
Kidney		
Hydroperoxides	-0.023	0.925
Protein carbonyls	-0.028	0.905

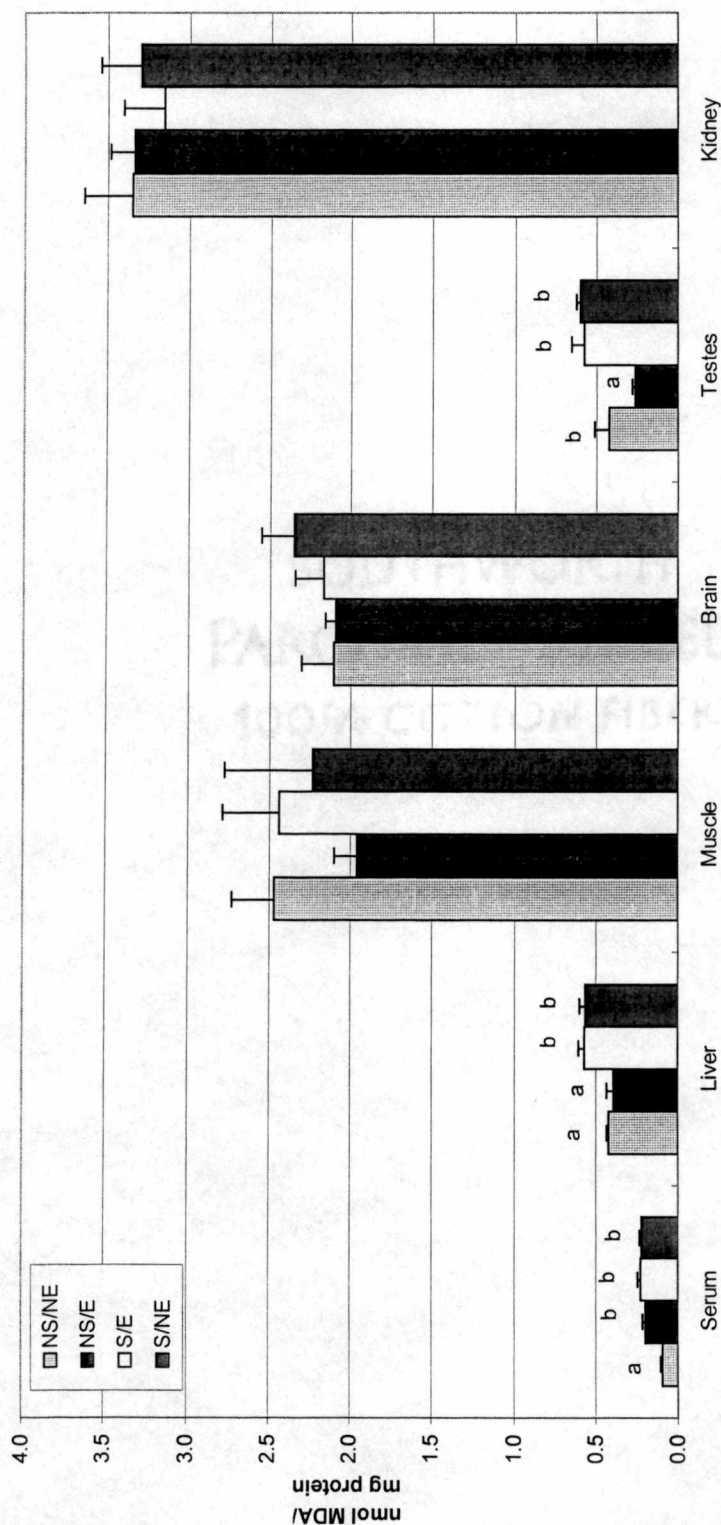


Figure 4-1 Effect of exercise and/or choline, carnitine and caffeine supplementation on serum and tissue TBARS

concentrations. The values are group means $\pm$ SEM ($n = 5$) and those columns within a tissue with no common superscript letter are significantly different ($p \leq 0.05$). (P-values for: serum < 0.001 , liver ≤ 0.002 , skeletal muscle ≤ 0.613 , brain ≤ 0.656 , testes ≤ 0.003 and kidney ≤ 0.935)

NS/NE = control, NS/E = exercised, S/E = supplemented and exercised, S/NE = exercised, S/NE = supplemented

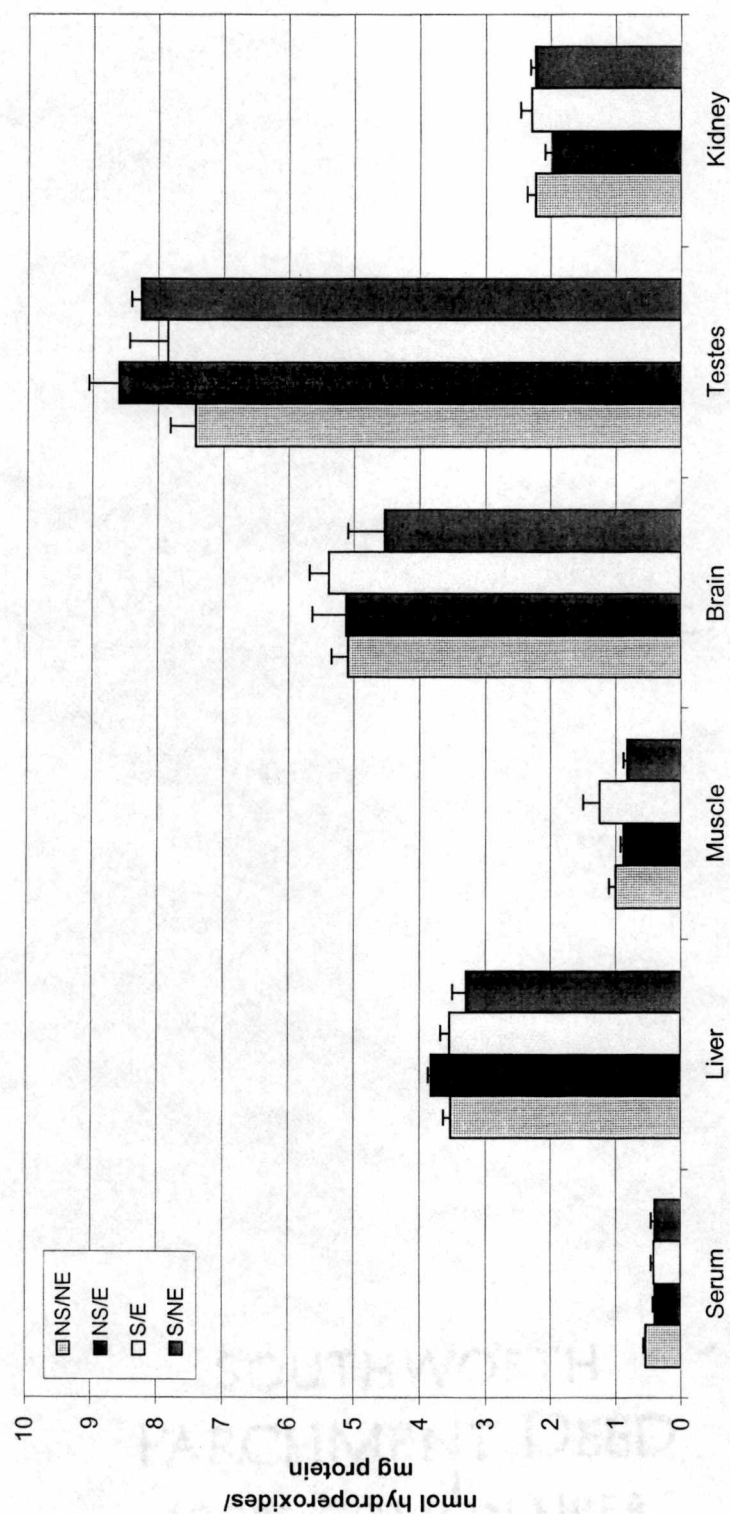


Figure 4-2 Effect of exercise and/or choline, carnitine and caffeine supplementation on serum and tissue

hydroperoxides concentrations. The values are group means $\pm$ SEM ($n = 5$) and those columns within a tissue with no common

superscript letter are significantly different ($p \leq 0.05$). (P-values for: serum ≤ 0.049 , liver ≤ 0.089 , skeletal muscle ≤ 0.218 , brain

≤ 0.466 , testes ≤ 0.248 and kidney ≤ 0.357)

NS/NE = control, NS/E = exercised, S/E = supplemented and exercised, S/NE = supplemented

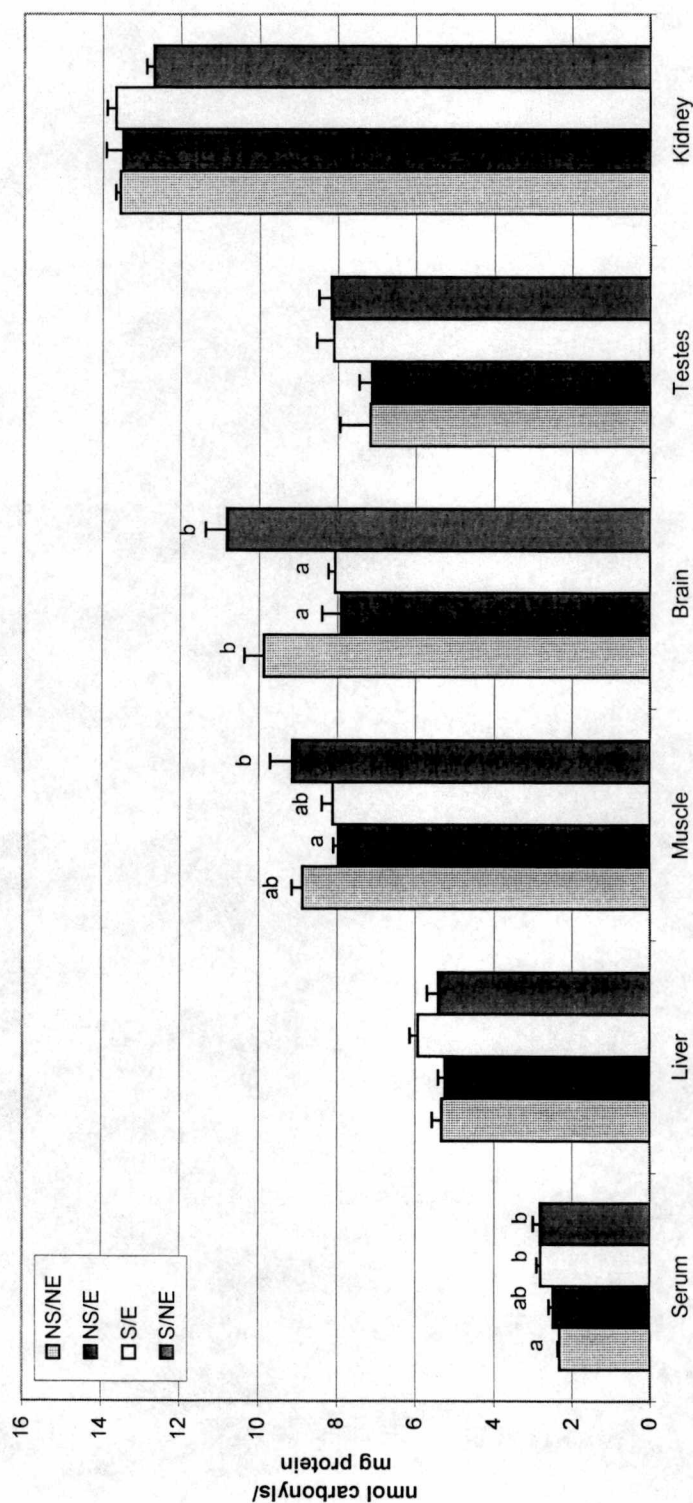


Figure 4-3 Effect of exercise and/or choline, carnitine and caffeine supplementation on serum and tissue protein

carbonyl concentrations. The values are group means $\pm$ SEM ($n = 5$) and those columns within a tissue with no common

superscript letter are significantly different ($p \leq 0.05$). (P-values for: serum ≤ 0.021 , liver ≤ 0.189 , skeletal muscle ≤ 0.074 , brain

≤ 0.001 , testes ≤ 0.303 and kidney ≤ 0.067)

NS/NE = control, NS/E = exercised, S/E = supplemented and exercised, S/NE = supplemented

Liver: Results summarized in **Table 4.2** reveal that supplemented rats had significantly higher TBARS ($p \leq 0.05$) but had no significant change in protein carbonyl or hydroperoxide concentrations. No significant change of TBARS concentration was observed with exercise alone. TBARS and protein carbonyls were positively correlated ($p \leq 0.05$) (**Table 4.7**).

Skeletal muscle: No observable affects were found in TBARS and hydroperoxide concentrations when the rats were exercised or supplemented (**Table 4.3**). However, animals that were only exercised had significantly lower protein carbonyl concentrations than animals given the supplement alone. A positive correlation was found between TBARS and hydroperoxides (**Table 4.7**)

Brain: The data for TBARS and hydroperoxide concentrations do not differ between the groups (**Table 4.4**). Protein carbonyl concentrations were decreased in both exercised groups but not in the group given the supplement alone ($p \leq 0.01$). A significant interaction of supplement and exercise was observed for protein carbonyls ($p \leq 0.001$).

Testes: The supplementation had no consequence on oxidative stress measured by TBARS concentration (**Table 4.5**). However, exercise alone significantly lowered TBARS concentration ($p \leq 0.01$). Hydroperoxides and protein carbonyl concentrations were not affected by exercise and/or supplement.

Kidney: No significant effect was observed for any marker by any treatment as shown in **Table 4.6**.

IV.2 Effects of Different Dietary Fats

The results of the effects of dietary fish oil, corn oil, safflower oil and palm oil on markers of oxidative stress in serum and liver of rats are presented in **Table 4.8 and 4.9** and illustrated in **Figure 4-4 to 4-6**. The serum TBARS concentrations were significantly lower in the rats fed a diet with palm oil compared to animals fed the other diets ($p \leq 0.01$). No significant effects of dietary fats were seen in concentrations of serum hydroperoxides and protein carbonyls.

In the fish oil group hepatic TBARS concentrations were significantly higher compared to the other groups ($p \leq 0.001$). The concentrations of hepatic hydroperoxides were significantly higher in the group fed fish oil compared to corn oil and palm oil ($p \leq 0.05$). Both the TBARS and hydroperoxides concentrations were the lowest in the palm oil group, which had the highest concentrations of protein carbonyls. However, concentrations in the palm oil group were not significantly different from the safflower oil and corn oil groups. The fish oil group had significantly lower concentrations of protein carbonyls than the palm oil group. Positive correlations between TBARS and hydroperoxides in serum and liver ($p \leq 0.01$) were observed (**Table 4.10**). Additionally, a negative correlation of hepatic TBARS and protein carbonyls ($p \leq 0.01$) was observed (**Table 4.10**).

Table 4.8

Comparison of serum TBARS, hydroperoxide and protein carbonyl concentrations in rats fed different dietary fats for 8 weeks¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/mg protein		
Fish oil ²	0.09 ± 0.01 ^{b3}	0.32 ± 0.11	2.60 ± 0.09
Safflower oil ²	0.12 ± 0.01 ^b	0.36 ± 0.04	2.12 ± 0.20
Corn oil	0.09 ± 0.02 ^b	0.48 ± 0.11	2.28 ± 0.25
Palm oil	0.06 ± 0.01 ^a	0.27 ± 0.04	2.47 ± 0.07

¹ Values are means ± SEM, (n = 6) except ² (n = 5) and ³ (n = 4), and those bearing different superscript letters within a column are significantly different at p ≤ 0.05

Table 4.9

Comparison of hepatic TBARS, hydroperoxide and protein carbonyl concentrations in rats fed different dietary fats for 8 weeks¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/ mg protein		
Fish oil ²	2.82 ± 0.34 ^b	6.07 ± 1.12 ^b	4.32 ± 0.24 ^a
Safflower oil ²	1.70 ± 0.19 ^a	5.01 ± 0.80 ^{ab}	4.81 ± 0.30 ^{ab}
Corn oil	1.72 ± 0.11 ^a	3.59 ± 0.30 ^a	4.71 ± 0.04 ^{ab}
Palm oil	1.34 ± 0.05 ^a	2.97 ± 0.30 ^a	5.12 ± 0.21 ^b

¹ Values are means ± SEM, (n = 6) except ² (n = 5), and those bearing different superscript letters within a column are significantly different at p ≤ 0.05

Table 4.10

Correlation coefficients (R) between TBARS and hydroperoxides or protein carbonyl concentrations in serum and liver of rats fed different dietary fats

	R	P-value
Serum		
Hydroperoxides	0.550	0.010
Protein carbonyls	0.063	0.785
Liver		
Hydroperoxides	0.642	0.001
Protein carbonyls	-0.557	0.007

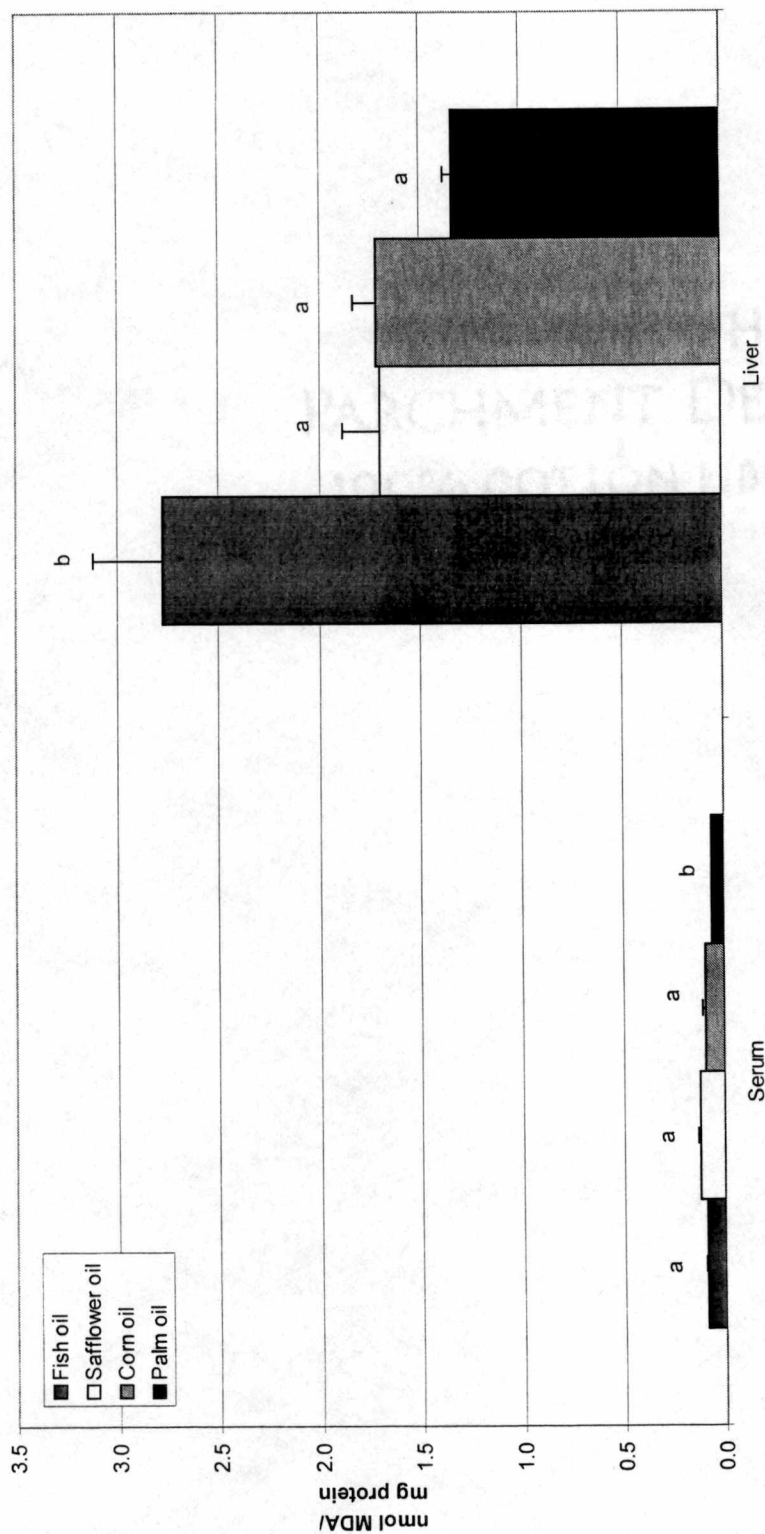


Figure 4-4 Effect of different dietary fats on serum and liver TBARS concentrations. The values are group means $\pm$ SEM ($n=5$), except serum of fish oil group ($n=4$) and the corn and palm oil groups ($n=5$) and those columns within a tissue with no common superscript letter are significantly different ($p \leq 0.05$). (P-values: serum ≤ 0.004 , liver < 0.001)

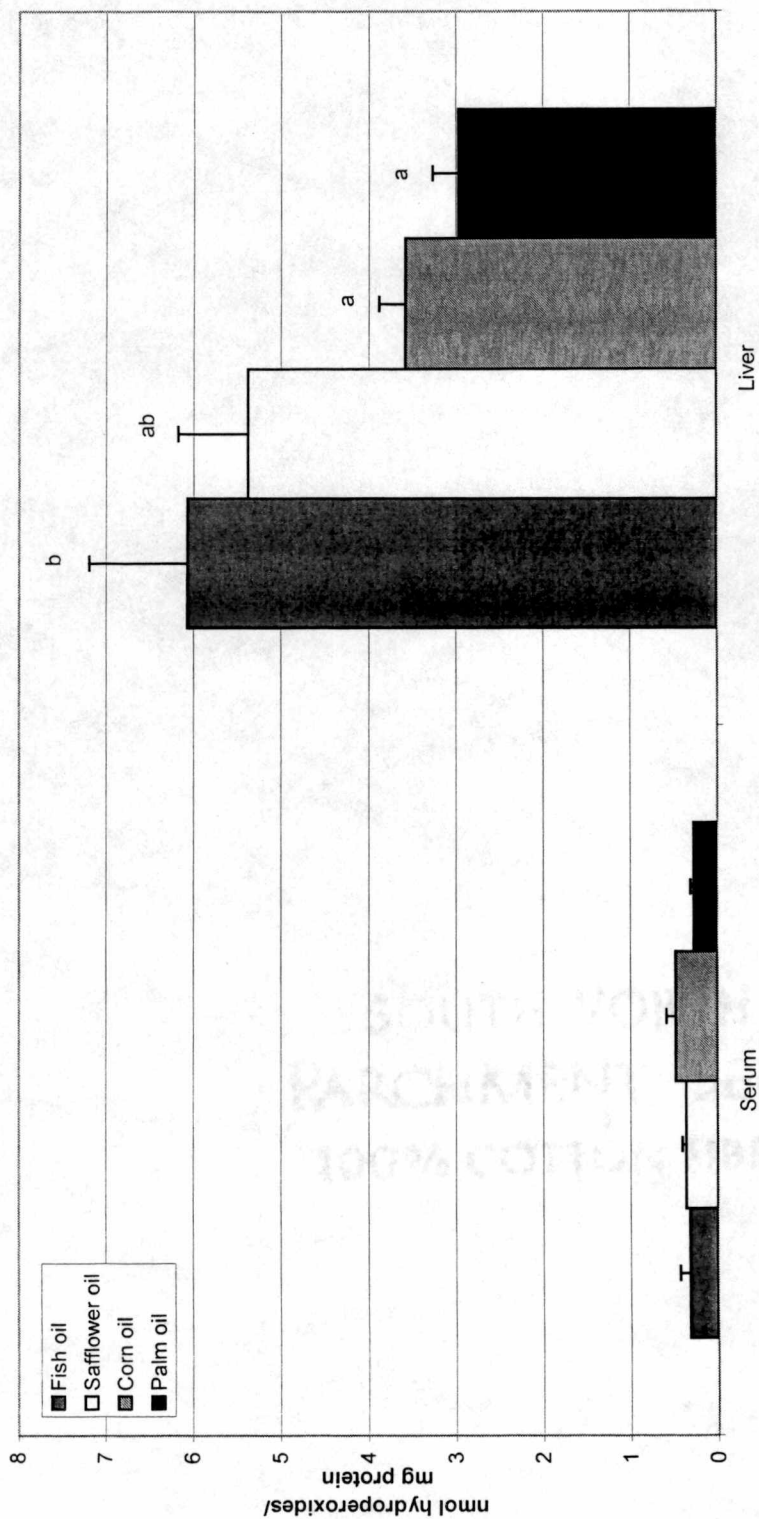


Figure 4-5 Effects of different dietary fats on serum and liver hydroperoxide concentrations. The values are group means $\pm$ SEM ($n = 5$), except for corn and palm oil groups ($n = 6$) and those columns within a tissue with no common superscript letter are significantly different ($p \leq 0.05$). (P-values: serum ≤ 0.068 , liver ≤ 0.017)

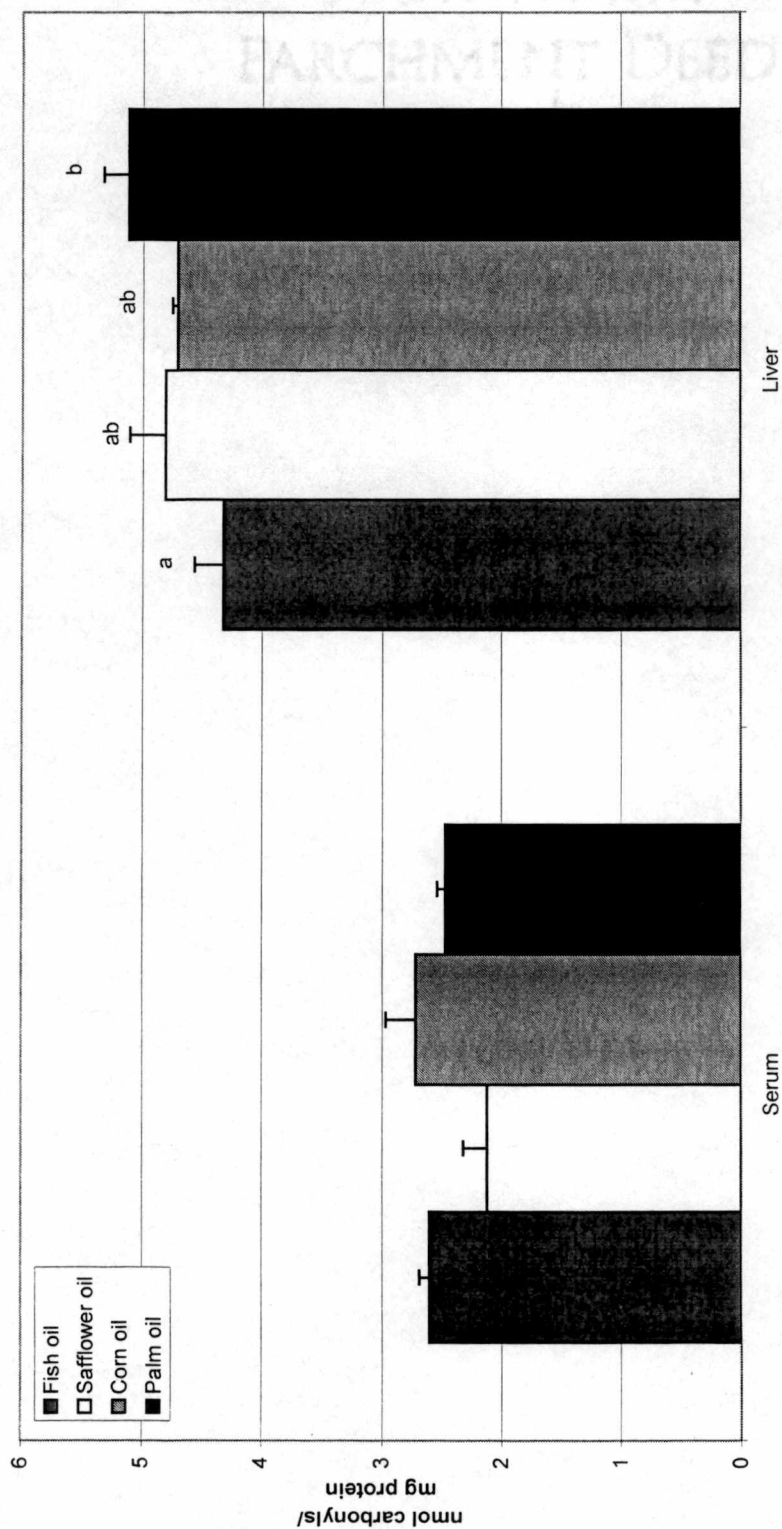


Figure 4-6 Effect of different dietary fats on serum and liver protein carbonyl concentrations. The values are group means $\pm$ SEM ($n = 5$), except for corn and palm oil groups ($n = 6$) and those columns within a tissue with no common superscript letter are significantly different ($p \leq 0.05$). (P-values for: serum ≤ 0.216 , liver ≤ 0.093)

IV.3 Effects of Cis and Trans Fatty Acids

The results of the effect of a diet containing predominately cis or trans fatty acids with or without an acute dose of ethanol on TBARS, hydroperoxides and protein carbonyl concentrations in serum are presented in **Table 4.11** and illustrated in **Figure 4-7 to 4-9**. The correlations of TBARS with hydroperoxides and protein carbonyl concentration are summarized in **Table 4.12**. The rats fed predominantly cis fatty acids and given an acute dose of ethanol had significantly higher concentrations of TBARS than either of the trans fatty acid fed groups ($p \leq 0.01$). In contrast, the cis fatty acid fed group without an acute dose of ethanol did not statistically differ from any group (**Figure 4-7**). Hydroperoxide concentrations were not significantly affected by the fatty acid isomers or an acute dose of ethanol (**Figure 4-8**). Protein carbonyl concentrations were significantly lower in rats fed trans fatty acids ($p \leq 0.05$) but an acute dose of ethanol showed no effect on the marker (**Figure 4-9**). No interactive effect was observed for fatty acid isomers and ethanol on any of the markers (**Table 4.11**).

Table 4.11

Comparison of serum TBARS, hydroperoxide and protein carbonyl concentrations in rats fed cis or trans fatty acids for 2 weeks with or without an acute dose of ethanol¹

	TBARS	Hydroperoxides	Protein carbonyls
	nmol/ mg protein		
CFA	0.13 ± 0.01 ^{ab}	10.02 ± 0.29	2.97 ± 0.08 ^b
CFA + EtOH	0.15 ± 0.01 ^b	9.57 ± 0.33	2.88 ± 0.06 ^b
TFA ²	0.11 ± 0.02 ^a	9.70 ± 1.57	2.54 ± 0.05 ^a
TFA + EtOH	0.10 ± 0.01 ^a	7.85 ± 0.75	2.51 ± 0.10 ^a
	Statistical significance ²		
Fatty acid	NS	NS	< 0.001
EtOH	0.001	NS	NS
Fatty acid x EtOH ³	NS	NS	NS

CFA = cis fatty acids, TFA = trans fatty acids, EtOH = ethanol

¹ Values are means ± SEM (n=6), except ² n = 4 and those bearing different superscript letters within a column are significantly different at $p \leq 0.05$

² Two-way ANOVA, $p \leq 0.05$; NS = not significant ($p > 0.05$)

³ Fatty acid and ethanol interaction

Table 4.12

Correlation coefficients (R) between TBARS and lipid hydroperoxides or protein carbonyl concentrations in serum of rats fed predominantly cis or trans fatty acids with or without an acute dose of ethanol

	R	P-value
Serum		
Hydroperoxides	0.313	0.081
Protein carbonyls	-0.144	0.433

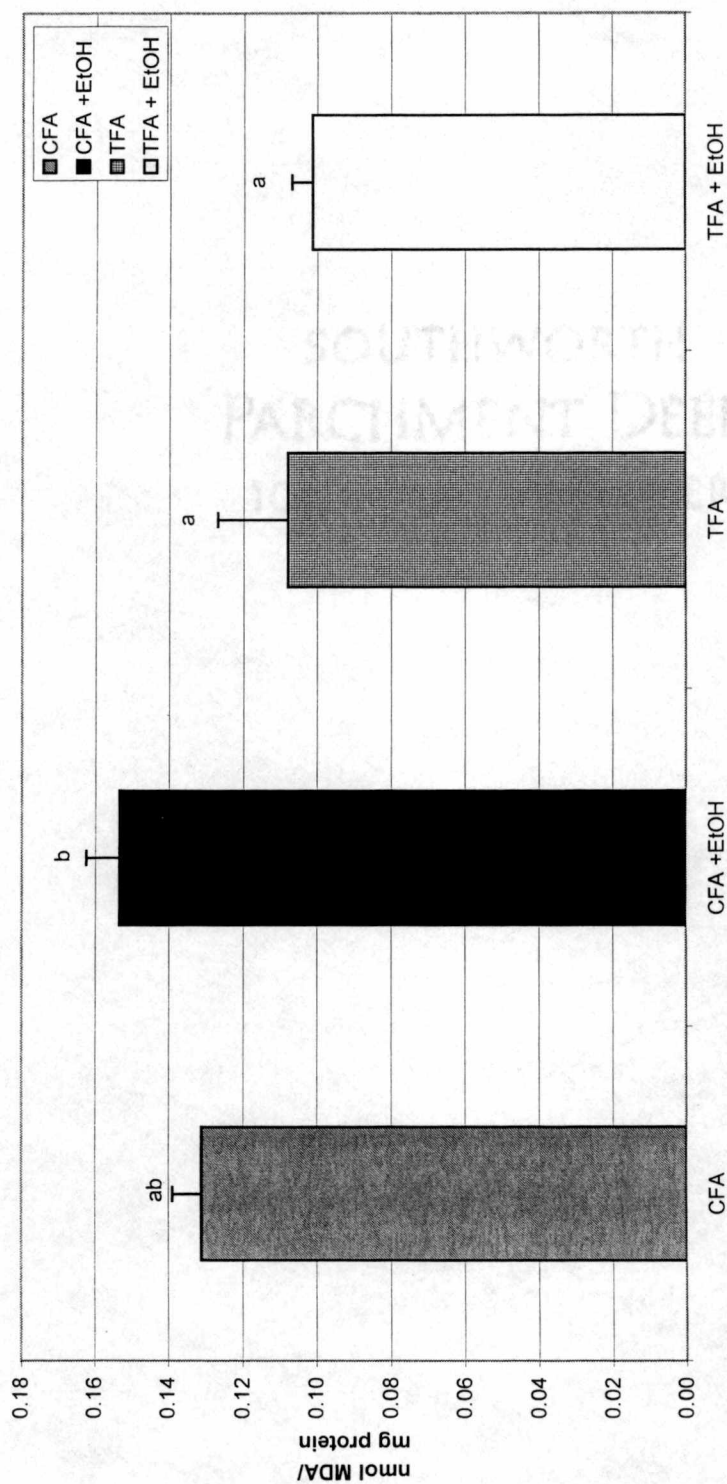


Figure 4-7 Effect of cis and trans fatty acids with or without an acute dose of ethanol on serum TBARS

concentrations. The values are group means $\pm$ SEM ($n = 6$, except TFA $n = 4$) and those columns with no common superscript letter are different ($p \leq 0.05$). (P-value: serum ≤ 0.001)

CFA = cis fatty acids, TFA = trans fatty acids, EtOH = ethanol

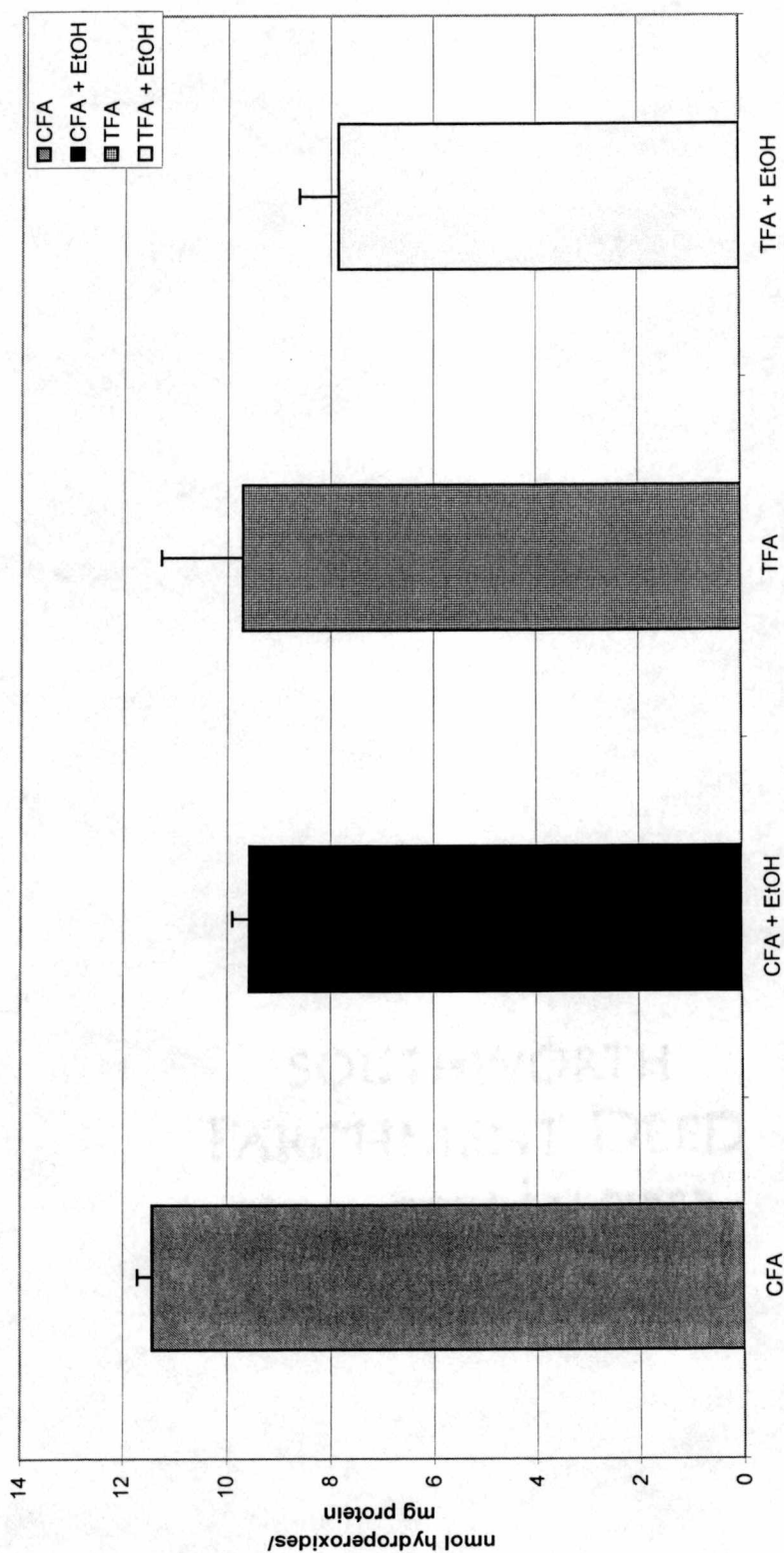


Figure 4-8 Effect of cis and trans fatty acids with or without an acute dose of ethanol on serum hydroperoxide concentration. The values are group means $\pm$ SEM ($n = 6$, except TFA $n = 4$) and those columns with no common superscript letter are significant different ($p \leq 0.05$). (P-value: serum ≤ 0.162)

CFA = cis fatty acids, TFA = trans fatty acids, EtOH = ethanol

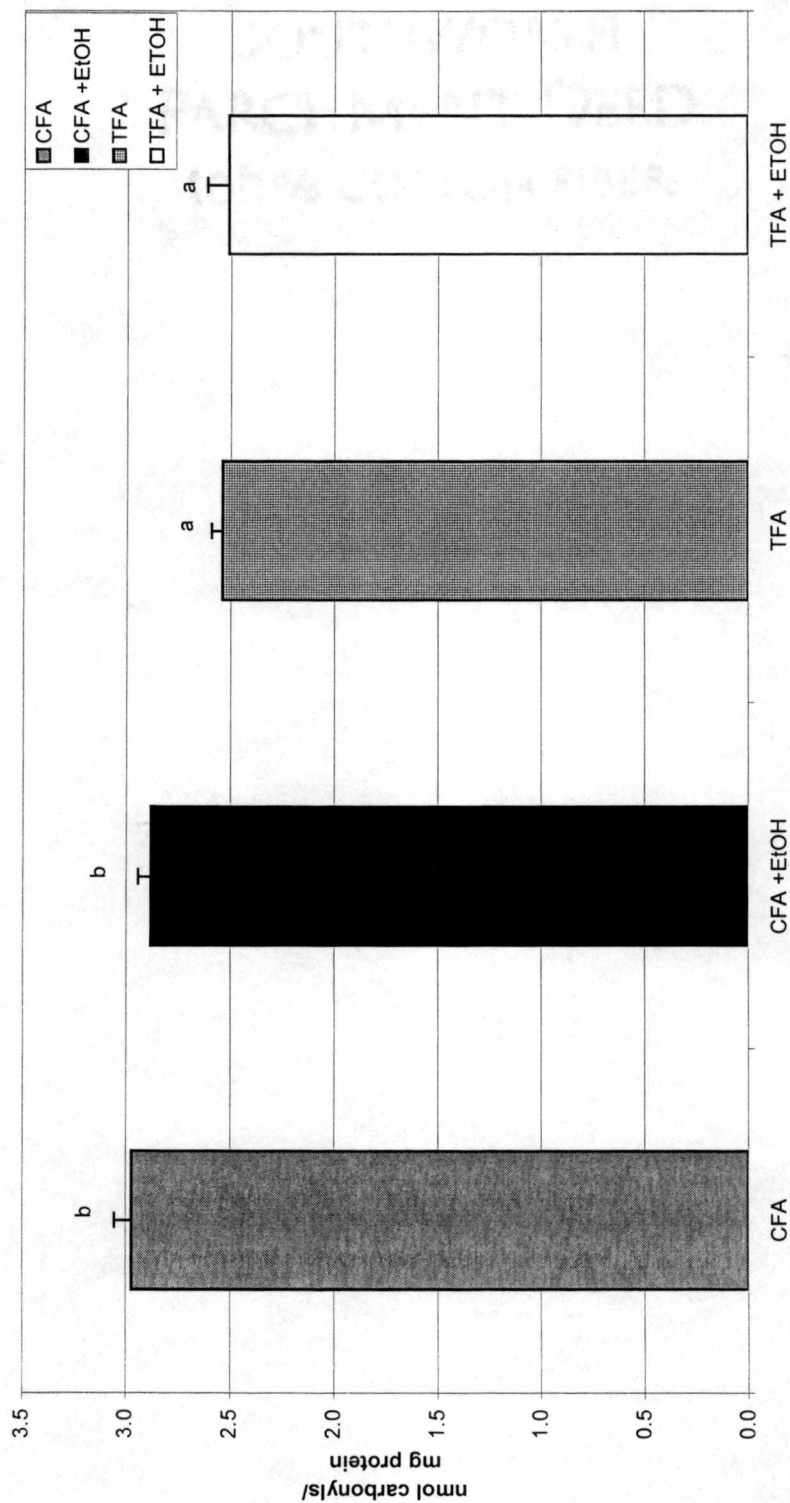


Figure 4-9 Effect of cis and trans fatty acids with or without an acute dose of ethanol on serum protein carbonyl

concentration. The values are group means $\pm$ SEM ($n = 6$, except TFA $n = 4$) and those columns with no common superscript

letter are significantly different ($p \leq 0.05$). (P-value: serum ≤ 0.005)

CFA = cis fatty acids, TFA = trans fatty acids, EtOH = ethanol

Chapter V

Discussion

In order to evaluate the relevance of different dietary fats or the use of a supplement combination of choline, carnitine and caffeine (CCC) on oxidative stress, TBARS, lipid hydroperoxides and protein carbonyls were determined. Each study will be separately discussed but some points of general nature are discussed below. In any study samples have to be stored until assayed. The length and conditions of storage may or may not affect the parameters being measured. It is important that this time be clearly indicated, which is not the case in the majority of the studies reported in the literature. In our studies, all samples were stored at -80°C . Those samples of the dietary fat study were stored for approximately four years (since 1996), the cis /trans fatty acid study samples for approximately 2 years (since 1998), and the samples of the choline, carnitine and caffeine study for one year (since 1999). The markers of oxidative stress were chosen with the consideration of storage time and that enzymes affect the concentrations of some markers such as glutathione (Anderson 1985, Carlberg and Mannervik 1985). No data were available regarding time-dependent changes in the markers used in our report.

In one study protein carbonyl concentrations were compared in three different muscles types (gastrocnemius, white and red quadriceps). The gastrocnemius muscle was stored at -80°C for 12 month prior to assay. The protein carbonyl concentrations were significantly lower in the gastrocnemius than in the other two muscle types. The authors

thought that it might be due to the length of storage, however they did not determine protein carbonyls prior to freezing or within days after freezing (Reznick et al. 1992). Therefore, it is difficult to conclude that length of storage caused lower protein carbonyl concentrations in the gastrocnemius muscle. Further, these authors found similar changes in protein carbonyl concentration, which were present among all muscles of the different treatment groups. It is, therefore, logical to conclude that differences in protein carbonyl concentration will be observed after samples have been stored for a prolonged period of time.

The products of lipid peroxidation, such as lipid hydroperoxides might decrease over time, due to autooxidation as these might get transformed into other products. TBARS are more likely increased with storage because they represent stable end products of lipid peroxidation and would be excreted into urine. It is generally believed that lipid hydroperoxides can contribute to the TBARS level during the assay procedure (Ohkawa et al. 1979, Moore and Roberts 1998). However, a recent report indicates that lipid hydroperoxides are not precursors of MDA in the TBA assay system (Jiang et al. 1991). It means that TBARS measured by the TBA assay are present in the tissue and not produced from lipid hydroperoxides during the assay procedure. Thus, the concentrations of these two entities are a reflection of their level in tissues. It is also understood that each tissue responds differently to oxidative stress and the markers may vary accordingly (Jenkins 2000). So, even if the autooxidation and enzymatic breakdown of oxidized products occur, as all samples of an experiment stored under identical condition, they must be affected in the identical manner and therefore, the effect of treatments should not be obscured (Reznick et al. 1994).

In our studies, we did not use any BHT or other antioxidants, as the addition of BHT in a preliminary trial exhibited no protective effect. There is no consistent use of BHT for TBARS assays reported in the literature. The concentrations of the markers in the present study were determined in the cytosolic fraction of the tissue homogenates and expressed as nmoles per mg protein. Thus, the use of BHT for the lipid hydroperoxide assay was not required (Wolff 1994). The majority of data available in literature has been presented as nmoles per g of tissue or has been investigated in the microsomal or the mitochondrial fraction and presented as nmoles per mg protein. This and the use of different methods make it sometimes difficult to compare our results with those in the literature.

V.1 Effects of Choline, Carnitine and Caffeine

The objective of this experiment was to determine the effect of the combination of choline, carnitine and caffeine supplements (CCC) and/or mild exercise on oxidative stress. The combination of supplements has been shown to reduce body fat mass as a result of an interactive effect on fat mobilization and fatty acid utilization (Hongu and Sachan 2000).

Results of certain studies indicate that carnitine supplementation improves exercise performance, however, there is controversy and data confirming this theory is debatable (Brass 2000). Choline supplementation has been shown to conserve carnitine and its concentration in tissues, particularly skeletal muscle, where it facilitates translocation of fatty acids into mitochondria (Dodson and Sachan 1996, Daily et al. 1998). Caffeine administration has been shown to increase free fatty acids and glycerol in

blood, indicating enhanced fat mobilization from adipose tissue (Pasman et al. 1995). The combination of supplements not only improved fat mobilization but also increased the concentrations of triglycerides in skeletal muscle and decreased serum triglyceride levels (Sachan and Hongu 2000).

Exercise has been demonstrated to reduce body fat mass similar to the CCC supplement (Hongu and Sachan 2000). In addition, the CCC supplement augmented exercise performance measured by VO_2 max (Sachan and Hongu 2000). Regular exercise has been shown to enhance concentrations of antioxidants in skeletal muscle and liver (Quiles et al. 1998). Under similar conditions lower concentration of oxidized DNA in skeletal muscle was observed without affecting TBARS and protein carbonyls (Radák et al. 1999). This indicates that oxidative stress induces antioxidant systems to cope with ROS (Pilger et al. 1997). It has been found that when trained and sedentary rats were put to exhaustive exercise, markers of oxidative stress were lower in the trained than sedentary rats (Oh-Ishi et al. 1997). The reduction in body fat mass and the increase in fat mobilization in the CCC supplemented rats were similar to the changes in the exercised animals (Hongu and Sachan 2000, Sachan and Hongu 2000). This follows the argument that the supplements and exercise should exert a similar effect on the antioxidant systems.

On the other hand, it might be expected that increased metabolic activity and thus enhanced fatty acid oxidation increases oxidative stress in the system. Our data show an increase in serum concentrations of TBARS and protein carbonyls but no changes in lipid hydroperoxides. Similar results were seen in the exercised group. There was no significant change in markers of oxidative stress in skeletal muscle of supplemented as well as exercised animals compared to controls. This may be due to enhanced antioxidant

levels in skeletal muscle following exercise (Quiles 1998) and carnitine supplementation (Kalaiselvi and Panneerselvam 1998). Another reason might be the stabilizing effect of carnitine on membranes (Palmieri et al. 1994, Ronca et al. 1994). An increase in TBARS concentration in liver is consistent with the increase of fatty acid oxidation in liver indicated by a rise of serum β -hydroxybutyrate (Sachan and Hongu 2000).

That markers of oxidative stress increased in serum but affected tissues less might be due to the age of rats. Supplementation with carnitine alone has been shown to significantly improve antioxidant status in old but not in young rats. Our results might suggest that the antioxidant activity of carnitine in our young rats helped to protect tissues from oxidative damage produced by an increase in metabolic activity caused by choline, carnitine and caffeine together.

V.2 Effects of Different Dietary Fats

Dietary fatty acid composition is a modulator of lipid peroxidation in serum, lipoproteins and tissues (L'Abbe et al. 1991, Scaccini et al. 1992, Haegele et al. 1994, Quiles et al. 1999). We evaluated the relevance of the dietary fatty acid composition on oxidative stress as measured by TBARS, lipid hydroperoxides and protein carbonyls in serum and liver of rats fed different dietary fats.

Our results are consistent with data reported by L'Abbe et al. (1991) and show that the fish oil diet increased liver TBARS levels approximately 1.5 fold compared to the corn oil diet. Similar to Quiles et al. (1994) TBARS and lipid hydroperoxide concentrations in serum (1999) and liver were increased with a more polyunsaturated diet. Our results indicated the highest concentration of lipid peroxidation in rats fed the

fish oil diet, which is in agreement with the results published by Garrido et al. (1989). Their results have shown that the membranes had an increase in eicosapentaenoic acid and docosahexaenoic acid content when the animals were fed fish oil compared to corn oil. Additionally, the membrane susceptibility to oxidative stress was higher.

Venkatraman et al. (1998) were not able to show significant differences in TBARS concentrations when rats were fed either corn or fish oil. However, the authors mentioned that vitamin E was three fold higher in both diets and might have caused an adequate protection against lipid peroxidation.

No significant difference in the oxidative markers between the serum of animals fed safflower oil and fish oil was observed in our study. These results are consistent with the data from a human study, in which erythrocytes had no significant difference in TBARS concentrations when given a supplement containing either safflower or fish oil (Mills et al. 1995). Additionally, it should be considered that Ohkawa et al. (1978) demonstrated that linoleic acid improved its reactivity during the TBARS assay when the pH was increased from 3.5 to 4. This might influence the results for safflower oil and corn oil treated rats as they have significantly more linoleic acid incorporated into their membranes (Mills et al. 1995, Venkatraman et al. 1998). Our results indicate that palm oil leads to significantly lower lipid peroxidation in liver and serum. It is consistent with data reported by Nesaretnam et al. (1993), who found that palm was able to significantly reduce TBARS levels in liver compared to corn oil.

Overall, the degree of lipid unsaturation determines the magnitude of lipid peroxidation in rat liver. Our data show a significant negative correlation between protein carbonyls and TBARS in liver. The palm oil group had significantly higher

concentrations of liver protein carbonyls than the fish oil group. This could be because radicals preferably attack unsaturated lipids and thus spare proteins. When dietary unsaturated fatty acids increase, there is corresponding a change in the fatty acid composition of cell membranes, which are a target of lipid peroxidation. All animals of the study were given an oral dose of ethanol 72 and 24 hours prior to killing. An acute dose of ethanol has been shown to increase urinary TBARS concentrations within 24 hours (Moser et al. 1993); therefore serum TBARS concentrations must have increased, too. Additionally, in vitro studies with brain mitochondria have shown that fatty acid ethyl esters produce more lipid hydroperoxides than their corresponding fatty acids and there was an increase in hydroperoxide concentration with increasing degree of unsaturation (Bondy and Marwah 1995). While ethanol metabolism is enhanced by unsaturated fatty acids, it is retarded by saturated fatty acids (Cha and Sachan 1994). Therefore; the acetaldehyde load will be higher in the livers of animals fed unsaturated fatty acids than saturated fatty acids fed animals. This may have contributed to higher TBARS and hydroperoxides in the livers of animals fed fish oil, corn oil or safflower oil detected in our study.

V.3 Effects of Cis and Trans Fatty Acids

Since unsaturated fatty acids have greater propensity to oxidative stress, the question was if cis and trans fatty acids have a different impact on markers of oxidative stress. Although still controversial, it is accepted that trans fatty acids do not adversely affect mortality or serum cholesterol (Alfin-Slater et al. 1957). Many studies, including those in our laboratory have shown that trans fatty acids are incorporated into membranes

similar to cis isomers (Emken 1983); linoleic acid and elaidic acid were significantly higher in rats fed trans fatty acids (Mong 1999). Our results indicate less oxidative stress in the trans fatty acids fed animals; however, significant differences were seen only for the protein carbonyls. Overall, lipid peroxidation in serum was similar, regardless of the fatty acid isomer in the diet. This may be due to the presence of double bonds and not the geometry of the double bounds.

Besides a slight increase in serum TBARS of the cis fatty acid group, acute ethanol administration had no significant affect on the oxidative stress markers used in our study. Although the increase in blood ethanol clearance, reported by Mong (1999), suggested that oxidative damage might increase, no significant changes in oxidative stress markers were observed. It is likely that this due to a lower absorption rate of ethanol in rats fed trans fatty acids (Mong 1999).

Chapter VI

Conclusion

From our results we conclude that dietary fats, a combination of choline, carnitine and caffeine supplements or exercise training effectively modulate oxidative stress. The supplement combination significantly increased markers of oxidative stress in serum, but not in tissues. Although fat mobilization was similar in exercised and supplemented rats, the markers of oxidative stress in tissues were different. Perhaps, tissues have antioxidant systems to protect against free radicals generated by enhanced metabolic processes and serum transports damaged molecules from the tissues. Animals fed a poly-unsaturated fatty acid rich diet were predisposed to higher lipid peroxidation than animals fed a diet rich in saturated fat. However, protein oxidation was higher in the animals fed a saturated fatty acid diet. Diets rich in trans isomers produced less oxidative stress compared to cis fatty acid diet. Further, we conclude that markers of oxidative stress in serum are not necessarily indicators of oxidative stress in the whole system. Also one marker may change differently than another in the same system.

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Appendix

Appendix 1

Composition of the experimental diets of the cis and trans fatty acid study

Ingredient	Dietary group	
	cis fatty acid	trans fatty acid
	g/kg	
Casein	200	200
DL-Methionine	3	3
Corn starch	150	150
Sucrose	450	450
Cellulose	50	50
Vitamin mix (AIN 76)	10	10
Mineral mix (AIN 76)	35	35
Choline bitartrate	2	2
Corn oil ^a	52	-
Olive oil ^a	37	-
Butter ^{2a}	14	1.27
Margarine ^{3a}	-	110.73
Elaidic acid ^a	-	12

¹ Adapted from the doctoral dissertation of M-S. Mong, 1999

² Butter: 113 g of butter contains 92 g of fat; therefore, there are about 81% fat in butter.

³ Margarine: 113 g of margarine contains about 91 g of fat; therefore, there are about 80% fat in margarine.

^a Differences presented in fat sources are due to their composition of fatty acids, which are balanced between both experimental diets.

Vita

Maike B. Johnsen was born in Cologne, Germany, on June 5, 1974. She attended high school at Paul-Klee-Gymnasium Overath, where she graduated in 1993. During her studies at the Rheinische-Friedrich-Wilhelms-University Bonn, Germany, she participated in an exchange program with the University of Tennessee, Knoxville. She graduated with a Bachelor of Science in Nutrition in 1998. She entered the Masters program in Nutrition at University of Tennessee at Knoxville in 1999 where she is graduating with a MS in Nutrition in May 2001.